PROCEEDINGS

OF THE

SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE

VOL. 32. JANUARY, 1935. No. 4.

New York Meeting.

New York Academy of Medicine, January 16, 1935.

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Ultrafiltration of the Virus of Vesicular Stomatitis.

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Galloway and Elford[1] recently filtered the virus of vesicular stomatitis through graded collodion membranes and found that the virus passed through membranes which had an average pore diameter of 160 mμ or greater, but was completely held back by those which had an average pore diameter of 130 mμ or less. From the analysis of their results they estimated the size of the virus particles to be 70-100mμ. They used the vesicular fluid from the pads of the feet of guinea pigs as a source of virus, and tested their filtrates for the presence of virus by intradermal inoculation into the pads of guinea pigs. Two different strains of the virus, serologically distinct, were studied, but no difference in the particle size was found.

* C represents a complete, P a preliminary manuscript.

1 Galloway, I. A., and Elford, W. J., *Brit. J. Exp. Path.*, 1933, **14**, 400.

Recently, Olitsky, Cox, and Syverton[2, 3, 4] have shown that mice are highly susceptible to this virus when inoculated intracerebrally and also that the virus can be maintained in tissue cultures. It was considered of interest to find out whether the particle size of the virus, derived either from the brain of infected mice or from the tissue cultures, is of the order of magnitude found by Galloway and Elford with virus from the vesicular fluid of guinea pig pads. To decide this point, the experiments here presented were carried out.

The collodion membranes used in these experiments were prepared according to the method of Elford,[5] with certain minor modifications adopted by Bauer and Hughes.[6] Infected mouse brains or tissue cultures were used as source of virus. The brains were ground in a mortar and suspended in a diluent consisting of equal parts of hormone broth, ascitic fluid, and distilled water. The concentration of the brain tissue in the suspension varied from one to 2% by weight. The suspension was centrifuged, and the supernatant fluid was passed first through a Seitz filter and then through a series of membranes of varying porosity. When the virus grown in tissue culture was used, 20 cc. of hormone broth and a similar amount of ascitic fluid were added to 60 cc. of the tissue culture. The mixture was centrifuged, and portions of the supernatant fluid were passed through a series of graded collodion membranes without preliminary filtration through a Seitz filter. All filtrations were carried out under positive pressure of nitrogen. In most of the filtrations the pressure was 100 cm. Hg., and in only a few instances were 2 atmospheres applied. The effective filtration area of each membrane was about 5 sq. cm., and the amount of filtrate collected from each membrane varied from 5 to 11 cc. The presence of virus in filtrates was tested in mice by intracerebral inoculation. A group of 6 mice was used for testing each filtrate, and the amount injected into each mouse was 0.03 cc. The virus content of the Seitz filtrate, or when tissue culture was used the unfiltered portion of the virus-containing material, was determined by titration in mice, using 4 or 6 mice for each dilution.

Two strains of the virus, the "Indiana" and the "New Jersey"

[2] Olitsky, P. K., Cox, H. R., and Syverton, J. T., *J. Exp. Med.*, 1934, **59**, 159.

[3] Cox, H. R., and Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 653, 654.

[4] Cox, H. R., Syverton, J. T., and Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 896.

[5] Elford, W. J., *J. Path. and Bact.*, 1931, **34**, 505.

[6] Bauer, J. H., and Hughes, T. P., *J. Gen. Physiol.*, 1934, **18**, 143.

strains, were studied. These were the same strains as those which had been used by Galloway and Elford in their studies, and they were sent us through the kindness of Dr. W. E. Cotton of the U. S. Bureau of Animal Industry. A total of 8 filtration experiments were carried out. In 5 of these the mouse-brain virus of the "Indiana" strain was used, in 2 the mouse-brain virus of the "New

TABLE I.
Filtration Experiment with Mouse-Brain Virus, "Indiana" Strain.

No. Membrane	Ave. pore diameter mμ	Amt. of filtrate collected cc.	Results of inoculation of filtrate in mice.*	Titration of stock filtrate	
				Dilution	Results*
205	180	10	6/6	10^{-2}	4/4
97	170	10	6/6	10^{-3}	3/4
173	160	10	6/6	10^{-4}	1/4
203	150	10	6/6	10^{-5}	0/4
181	150	10	6/6		
174	140	10	6/6		
206	130	10	0/6		

* The numerator represents the number of mice that succumbed to infection; the denominator, the number of mice employed in the test.

TABLE II.
Filtration Experiment with Mouse-Brain Virus, "New Jersey" Strain.

No. Membrane	Ave. pore diameter mμ	Amt. of filtrate collected cc.	Results of inoculation of filtrate in mice.*	Titration of stock filtrate	
				Dilution	Results*
137	160	10	6/6	Undiluted	6/6
203	150	10	2/6	10^{-1}	6/6
174	140	10	4/6	10^{-2}	5/6
215	140	10	0/6	10^{-3}	3/6
206	130	10	0/6	10^{-4}	1/6
259	130	10	0/6		
221	120	10	0/6		
219	120	10	0/6		

* The numerator represents the number of mice that succumbed to infection; the denominator, the number of mice employed in the test.

TABLE III.
Filtration Experiment with Tissue Culture Virus, "New Jersey" Strain.

No. Membrane	Ave. pore diameter mμ	Amt. of filtrate collected cc.	Results of inoculation of filtrate in mice.*	Titration of unfiltered suspension	
				Dilution	Results*
137	160	8	6/6	10^{-1}	4/4
173	160	6	6/6	10^{-2}	4/4
181	150	8	6/6	10^{-3}	3/4
174	140	5	0/6	10^{-4}	2/4
206	130	8	0/6	10^{-5}	0/4
138	120	6	0/6		

* The numerator represents the number of mice that succumbed to infection; the denominator, the number of mice employed in the test.

Jersey" strain, and in one the tissue culture virus of the latter strain. Three typical experiments are shown in Tables I, II, and III. It will be seen from these tables that the virus passed through all membranes which had an average pore diameter of 150 mμ or greater. The passage through the 140 mμ membranes was irregular, as in some of the experiments the filtrates proved infective, while in others they failed to produce infection. The filtrates of membranes which had an average pore diameter of 130 or less gave uniformly negative results. The filtration end-point, therefore, is considered to be approximately 140 mμ. These results are in close agreement with those obtained by Galloway and Elford.

Summary. The filtration end-point of the virus of vesicular stomatitis, or the average pore diameter of the finest membrane passing the virus, was found to be approximately 140 mμ. Two immunologically distinct strains of the virus, the "Indiana" and the "New Jersey" maintained either in tissue culture or in mouse brain, were studied, and the filtration end-point was found to be the same irrespective of the source or serological type of the virus.

Our results confirm those of Galloway and Elford, who found that the virus passes through collodion membranes which have an average pore diameter of 160 mμ but is held back by those of 130 mμ. From their results they estimated the particle size of the virus to be between 70 and 100 mμ.

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Early Diagnosis of Rabies by Mouse Inoculation. Measurement of Humoral Immunity to Rabies by Mouse Protection Test.

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A method for early and reliable diagnosis of rabies by animal inoculation and a protection test for measuring humoral immunity to the virus may become feasible by the use of highly susceptible strains of mice. Ordinarily the injection of brain tissue from rabid animals into rabbits or guinea pigs incites the disease irregularly and after incubation periods of 2 to 8 weeks. Mice as test animals have proved in the past even less satisfactory.[1] Mice especially bred for

[1] Koch, J., *Kolle-Kraus-Uhlenhuth. Handb. der Path. Mikroorg.*, 1930, **8**, 547.

high susceptibility to neurotropic viruses, however, are more sensitive and uniform in their response to the rabies street virus.

The diagnostic test for rabies by mouse inoculation is carried out in the following manner: Fresh dog brain containing Negri bodies* is seared and dissected aseptically to expose Ammon's horn. This area is removed and portions emulsified, diluted approximately 10 times, centrifuged, and injected into mice intracerebrally in 0.03 cc. quantities and intraperitoneally in ½ cc. quantities. Some of the mice are sacrificed from the 5th to 8th days, their brains removed, and impression transfers made from Ammon's horn and stained to determine the presence of Negri bodies. The remainder are observed for the appearance of characteristic weakness and paralysis of hind legs, prostration, and death. Final tests for Negri bodies are made on sick and prostrate mice.

To date, 40 dog brains received by the New York City Department of Health Laboratories have been tested. In 32 specimens, no Negri bodies were found and the test mice remained well for 30 days. In 7 specimens, Negri bodies were demonstrable. Of these, 2 specimens, Nos. 1 and 3, were injected intracerebrally into 6 and 19 mice respectively. The 6 mice receiving specimen No. 1 remained well 10 days, were weak or paralyzed on the 11th day, and prostrate or dead on the 12th day. All showed abundant Negri bodies in stained impression transfers. Of the 19 mice given specimen No. 3, 2 were killed on the 5th and 2 on the 6th days and examined for Negri bodies. A positive diagnosis could not be made definitely on these mice. The remaining 15 mice became weak or paralyzed on the 10th to 13th days and all contained Negri bodies. The other 5 specimens, Nos. 2, 19, 29, 41 and 42, were injected both intracerebrally and intraperitoneally. Examinations for Negri bodies were questionable on the 5th day and positive on the 6th day. All remaining mice became weak or paralyzed on the 7th to 9th days and died on the 10th to 15th days. In these mice Negri bodies were invariably abundant. Brains of 28 supposedly normal skunks, squirrels, cats, rats, etc., have also been tested with negative results. Of the total 68 specimens, 2 contained bacterial contaminants fatal to the test mice. This brief experience suggests that by the intracerebral and intraperitoneal injection of special mice, rabies may be diagnosed within 7 days.

* The assistance of Dr. W. H. Park and Miss A. Mann, of the New York City Department of Health Laboratories, is gratefully acknowledged. Specimens from possible cases of rabies sent to the City Laboratory for diagnosis were preserved in clean condition at low temperature and after being studied, one-half of the whole brain was brought here for further testing.

TABLE I.

Protective Effect of Serum from Individual Receiving Semple Anti-Rabic Treatment Against Rabies Virus.

Serum	Virus serum dilutions. 0.03 cc. per mouse intracerebrally.							
	10^{-1}		10^{-2}		10^{-3}		10^{-4}	
	No. of mice injected	Duration of life in days	No. of mice injected	Duration of life in days	No. of mice injected	Duration of life in days	No. of mice injected	Duration of life in days
Broth control	3	8, 9, 10	3	9, 10, 10	3	11, 13, 13	4	14, 15, 15, 15
Untreated—W	4	9, 10, 10, 10	4	9, 9, 10, 10	4	12, 13, 15, 16	4	15, 16, 16, S*
Treated—D	4	13, 13, 13, 15	4	13, 13, 16, S	4	S, S, S, S	4	S, S, S, S

* S = Remained well 30 days.

The virus-containing dog brains proved infective for these mice in doses of 10^{-5} gm. when injected intracerebrally and 10^{-1} gm. when injected intraperitoneally or subcutaneously. Two to 5 intracerebral passages of the virus in these mice reduced the incubation period to 6 days without change in number, size, or appearance of the Negri bodies; the intraperitoneal and subcutaneous titres increased to 10^{-3}. The virus traverses Seitz filters in 10^{-1} and 10^{-2} dilutions when treated in the manner described by Bauer and Hughes.[2]

A mouse protection test for the quantitative measurement of protective antibodies against rabies virus is being developed. Brains from mice prostrate 8 to 9 days after intracerebral injection of mouse brain virus in the 2nd to 5th passage are emulsified, diluted, centrifuged, diluted again, combined with equal parts of test sera for 2 hours at 37°C. and 2 hours at 23°C., and then injected in 0.03 cc. quantities intracerebrally in mice in dilutions of 10^{-1} to 10^{-5}. The duration of life of the injected mice is recorded in days.

Five tests with serum from one individual 1½ years after receiving the last of 3 courses of Semple anti-rabic treatment and with sera from 4 untreated individuals have given uniform results. The protocol of the first test is summarized in Table I. Data thus far show that sera from the 4 untreated individuals do not protect mice against an intracerebral injection of 10^{-6} gm. of mouse brain virus of 4 different strains but that serum from the treated person does protect against at least 100 lethal doses of these same strains.

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Numerical Relations of an Unstable Variant of Salmonella Aertrycke.

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Although many bacteriologists have encountered variants which were difficult to stabilize, most have felt that with sufficient care and repeated selection, any variant could be obtained in a stable form. In our studies on colonial forms of *Salmonella aertrycke* certain variants were encountered which lacked stability. A variant of this

[2] Bauer, J., and Hughes, T. P., *J. Gen. Physiol.*, 1934, **18**, 143.

type cannot be obtained free from another variant to which it constantly gives rise. The variation of these cultures was not haphazard but seemed to be governed by some fixed rule, the mechanism of which we have attempted to investigate.

We studied a strain MT2C-R, a rough variant derived from the typical smooth strain of *S. aertrycke*. A typical colony of this variant on infusion agar is low, rough and of medium size. When one was plated on infusion agar about 83% of the colonies derived from it were identical with the parent type. The other 17% were smooth and dome shaped and about 1/10 the diameter of the parent colony (MT2C-S). On continued daily subculture of these rough colonies on infusion agar plates, the percentage of rough colonies remained constant at 83% ± 5%. The S colonies when plated in the same way always yielded 100% S colonies.

When a 24-hour *broth* culture of an R colony was streaked on an agar plate, the percentage of R colonies fell to 33% or less; but each of these R colonies when subsequently plated directly, yielded the original 83%. When, however, the broth culture from the R colony was inoculated into broth a second time the second broth culture, on plating, yielded only S colonies.

Thus the percentage of R forms obtained on streaking out a broth culture of an R colony is less than that obtained by streaking the R colony directly on a second agar plate. Nevertheless, the intermediate broth passage in no way affects the inherent tendency of an R organism to give rise to a colony containing a constant ratio of R and S cells (about 83% R and 17% S).

Although the percentage of R colonies on a plate made from an R colony was found to be constant under constant conditions, a change in the conditions (*e. g.*, pH, temperature, peptone, and the like) gave rise to changes in the percentage of R colonies; but this percentage remained constant at the new level as long as the new conditions were kept constant.

The constancy of behavior of the cultures described above suggested that these phenomena might lend themselves to a simple mathematical analysis.

Let N = total number of cells per unit volume of culture.
R = number of R cells per unit volume of culture.
S = number of S cells per unit volume of culture.
T = time of growth.

Since S cells under the conditions of the experiment never give rise to R cells we may express the rate of increase of R cells during the exponential phase of growth by:

$$\frac{dR}{dT}=CR \qquad (1)$$

S cells, however, may arise from both R cells and S cells so we must write:

$$\frac{dS}{dT}=aS+bR \qquad (2)$$

Where a, b, and c are constants depending on the conditions of growth. $\frac{b}{b+c}$ represents the ratio of the number of S cells derived directly from R cells in an interval dT to the total number of cells derived directly from R cells in that interval.

If equations (1) and (2) are integrated and the integration constants determined by putting:

$$R=R_o \qquad S=S_o \qquad \text{when } T=0$$

we get:

$$R=R_oe^{cT} \qquad (3) \qquad\qquad c=\tfrac{1}{T}\log_e\tfrac{R}{R_o} \qquad (3')$$

$$S=\left(S_o-\frac{bR_o}{c-a}\right)e^{aT}+\frac{b}{c-a}R_oe^{cT} \qquad (4)$$

$$\frac{S}{R}=\left(\frac{S_o}{R_o}-\frac{b}{c-a}\right)e^{(a-c)T}+\frac{b}{c-a} \qquad (5)$$

If $c > a$ then equation (5) gives:

$$\operatorname*{Lim}_{T\to\infty}\frac{S}{R}=\frac{b}{c-a}=\text{a constant} \qquad (6)$$

This is evidently the condition when the organisms are grown on agar as described above. If we start with:

$$\frac{S_o}{R_o}=\frac{b}{c-a}=\operatorname*{Lim}_{T\to\infty}\frac{S}{R}$$

then adding equations (3) and (4) gives:

$$S+R=N=(S_o+R_o)e^{cT}=N_oe^{cT} \qquad c=\tfrac{1}{T}\log_e\frac{N}{N_o} \qquad (7)$$

but for a pure S culture we have:

$$a=\tfrac{1}{T}\log_e\frac{N}{N_o} \qquad (8)$$

then since $\frac{b}{c-a}$ is directly determinable from the final percentage of variants on an agar place we can directly determine the constants a, b, and c for growth on agar.

In broth S/R has been shown to increase as long as T increases, so we must have $a \gtreqless c$. If we assume* $a = b + c$ then adding equations (3) and (4) gives:

$$S + R = N = (S_o + R_o)e^{aT} = Ne^{aT} \qquad a = \tfrac{1}{T}\log \frac{N}{N_o} \qquad (9)$$

which is the same as we would get from a pure S culture.

Further, equation (5) becomes:

$$\frac{S}{R} = \left(\frac{S_o}{R_o} + 1\right)e^{bT} - 1 \qquad b = \tfrac{1}{T}\log_e\left(\frac{1 + S/R}{1 + S_o/R_o}\right) \qquad (10)$$

It is clear then that the constants can be determined independently in broth and on agar and that it could be possible to check the theory experimentally.

The preliminary experiments give following values for the constants:

	b	a	c	$\frac{b}{b+c}$ (primary ratio)
From S broth	—	1.26 ± .02	1.20 ± .02	.047
" R "	.059 ± .001	1.30 ± .02	1.24 ± .02	.045
" agar	.061 ± .006	1.04 ± .02	1.22 ± .02	.048

Since the experimental error is about 10% the agreement in the values of $\frac{b}{b+c}$ (representing the primary ratio) is probably better than the method allows. As closely as can be determined, this quantity remains a constant, under the different environmental conditions investigated.

We have here an organism which gives rise to R and S forms in a ratio which may be altered by varying the environment, *e. g.*, solid or liquid media. This modification of the ratio depends entirely on the growth rate of the individual daughter types. That is, the environment increases the relative growth rate of one with respect to the other. It in no way alters the primary rate of variation which is a numerical expression of the ability of the cell to give rise to 2 types of daughter cells in constant ratio.

It seems possible that the underlying mechanism of this type of variation is the same as that of the more commonly reported types of dissociation. If the assumptions on which the preceding calcu-

* If $a = c$ (9) becomes $S + R = S_o e^{aT} + bR_o e^{aT}$ (9′)
(10) becomes $S/R = S_o/R_o + bT$ (10′)
The data are not good enough to distinguish finally between these two assumptions but, $a = b + c$ was chosen because of its greater simplicity and a somewhat better check.

lations are based are correct, then we have here a type of culture whose composition may be deliberately changed in opposite directions by varying the environment, *i. e.*, solid or liquid media, without influencing the rate at which new variants arise from the culture. If the commoner forms of dissociation differ only quantitatively from the phenomena here described, then studies on bacterial dissociation must be concerned with 2 distinct phenomena: (1) The rate of origin of the new variant cells (primary rate of variation). (2) The environmental factors which make it possible for the new variant cells to grow to sufficient numbers to be detected in culture.

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Isolation of a Specific Ascorbic Acid (Vitamin C) Oxidase.

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An extract of Hubbard squash oxidizes both the synthetic and the natural ascorbic acid (vitamin C)* with great rapidity. This is due to an enzyme having an optimum pH of 5.83 to 5.96. It may be obtained and purified by extracting the squash (edible part) with twice its weight of 30% ethyl alcohol for 10 minutes. The centrifuged and filtered fluid is treated with an equal volume of acetone, which causes a yellow sticky substance to precipitate. This may be washed free of yellow pigment with acetone, dissolved in water and reprecipitated with acetone. A third precipitation yields a preparation, which after drying *in vacuo* over sulphuric acid has an activity 500 times that of the original extract.

This preparation is water soluble and gives slight protein tests. Alcohol and saturated solutions of neutral salts, however, do not precipitate it. It is digested (inactivated) by trypsin. A polysaccharide accompanies the enzyme in the above precipitation. We have found no way of removing it thus far.

This enzyme differs in various ways from the "hexoxidase" which v. Szent-Györgyi[1] discovered in cabbage leaves, *e. g.*, the

* We are indebted to Professor v. Szent-Györgyi for a sample of l-ascorbic acid and to the Hoffman La Roche Co. for some of their synthetic l-ascorbic acid (Redoxon).

[1] Szent-Györgyi, A., *Science*, 1930, **72**, 125; *J. Biol. Chem.*, 1931, **90**, 385.

hexoxidase is precipitated by saturated $(NH_4)_2SO_4$ solution; it oxidizes not more than about 25% of the substrate, whereas the enzyme of the squash oxidizes 100% very rapidly. Moreover, the kinetics of our preparation point to the presence of a single enzyme. Substances thus far tested, such as cysteine, tyrosine, glutathione, and phenols, are not affected. We suggest, therefore, that the enzyme responsible be designated "ascorbic acid oxidase".

It requires the presence of atmospheric oxygen, which plays the rôle of hydrogen acceptor. The oxidized ascorbic acid may be reduced to its original state by hydrogen sulphide. The enzyme is remarkably stable to dialysis, oxygen and carbon monoxid. Hydrogen sulphide, however, inactivates it.

For ascorbic acid estimation, Tillmans and associates'[2] 2,6-dichlorobenzenoneindophenol method was employed.

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Carcinomatous Changes in Virus-induced Papillomas of the Skin of the Rabbit.

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The Shope rabbit papilloma, a skin growth caused by a virus,[1] has been shown to possess the characters whereby tumors are recognized.[2] When given opportunity, as on implantation within the host, the growth frequently looks and behaves like a malignant neoplasm. The present report is concerned with instances in which skin papillomas caused by the virus have spontaneously become carcinomatous. The change has been noted in 5 of 10 domestic rabbits with growths existing 4 to 8 months.

During the early weeks of its development after virus inoculation the papilloma enlarges laterally, but later it is restricted by scar tissue and builds outwards only. At first it overlies the skin appendages, but these disappear after a time and it becomes bedded somewhat more deeply. The malignant change may first attract attention when a fissure exuding serosanguineous fluid opens in the midst

[2] Tillmans, J., Hirsch, P., and Hirsch, W., *Z. Untersuch. Lebensmittel*, 1932, **63**, 1.

[1] Shope, R. E., *J. Exp. Med.*, 1933, **58**, 607.

[2] Rous, Peyton, and Beard, J. W., *J. Exp. Med.*, 1934, **60**, 701.

of the papilloma; but more often there occurs a generalized, fleshy, discoid thickening of the base of the growth, which gradually raises the jagged, dry, papillomatous tissue some millimeters above the skin surface, and also bulges downwards. Soon the animal gnaws at this portion of the growth, opening in the one case a depressed ulceration with firm, gristly walls, and in the other laying bare a high, fungoid mass. On biopsy a squamous cell carcinoma is found, or an invasive, papillomatous, epithelial tumor, or most frequently, the 2 intermixed, with every gradation between them. The growth may for some weeks remain circumscribed and fungating, or it may rapidly extend under the skin, involving it and the muscle and becoming fixed upon the deep tissues. Some cancers less than 2 months old are already 5 cm. across and 2-3 cm. deep. As a rule they become infected with pus-producing organisms and the health of the host suffers, though most of our rabbits are still alive. Metastasis to a regional lymph-node (confirmed by section) has occurred once. Transplantation to the leg muscle of the host has resulted in a secondary, highly invasive, squamous cell carcinoma.

In rabbits carrying numerous discrete papillomas, as result of tattooing a virus fluid into the skin at many spots, nearly all of the growths undergo malignant changes within a few weeks of one another. The course of events has been followed by frequent biopsies. For a long time macrophages, lymphocytes and plasma cells have been assembling beneath the growth, and concurrently its epithelial folds have become less evenly ranked, its cells progressively more disordered in arrangement, and here and there proliferating tongues of them have thrust down a little further than the generality. Melanomatous growths cease to be pigmented. Frank invasion of the underlying tissue often begins at several places at once, and it occurs as a rule at points where focal bacterial infection has given rise to reactive connective tissue proliferation. The papillomatous structure may be retained even when the tumor has pushed down into the muscle; but more often the advancing processes progressively break up into irregular strands and groups of cells, and the growth becomes a frank, squamous cell carcinoma.

The virus engendering the Shope papilloma must be held primarily responsible for the carcinomas as well, but their proximal cause remains to be determined. In our previous work[3] the observation was several times made that newly engendered papillomas implanted in the leg muscles of the host promptly assumed the form of squamous cell carcinomas and invaded and replaced the indi-

[3] Rous and Beard, *loc. cit.*

vidual muscle fibres. This happened when there was associated with the growth a reactive connective tissue proliferation due to contaminating bacteria. It was plain that from an early period the cells of the papilloma possessed potentialities for malignant behavior. But rabbits appropriately tarred may develop carcinoma of the ear within 2 weeks;[4] and yet tar cannot be considered as the proximal cause of the cancer. Even if the Shope virus gave rise forthwith to carcinoma the view would still be tenable that it had done no more than provide the conditions requisite to a cancerous change of unknown cause. At the moment this much only seems certain: The papilloma virus gives rise to skin growths which for some time are benign tumors, though their cells have malignant potentialities. Gradually, by alterations which involve no discontinuity of form or behavior, the growths take on the character of typical carcinomas. Bacterial infection frequently acts to precipitate the change.

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Sanitary Significance of the Succession of Colon-Aerogenes Organisms in Feces.

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A study has been made of 57 specimens of feces from normal humans, from which by direct plating, 1454 strains of bacteria have been isolated. Eight of the 57 were stored at 37°C. and plated 60 times during the period of their viability, 2 to 3 months, yielding 592 strains. Duplicates of these 8 and 4 others of the 57 were kept in the ice box and on 162 platings have yielded 1086 strains. The period of viability for cold stored suspensions varies but may exceed 15 months. The feces were suspended in sterile saline to a heavy turbidity and platings have been made from these suspensions. The procedure diagrammed has been adopted. It provides for adequate purification of strains isolated, permits a comparison of the reactions of purified and original strains, and utilizes the tests now considered most significant for the group. Recognition of lactose-deficient organisms in normal feces[1] has led to the inclusion in the series of all organisms typical of the group which ferment dextrose.

[4] Bittman, O., *Z. f. Krebsforsch.*, 1925, **22**, 278.

[1] Parr, L. W., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 1019.

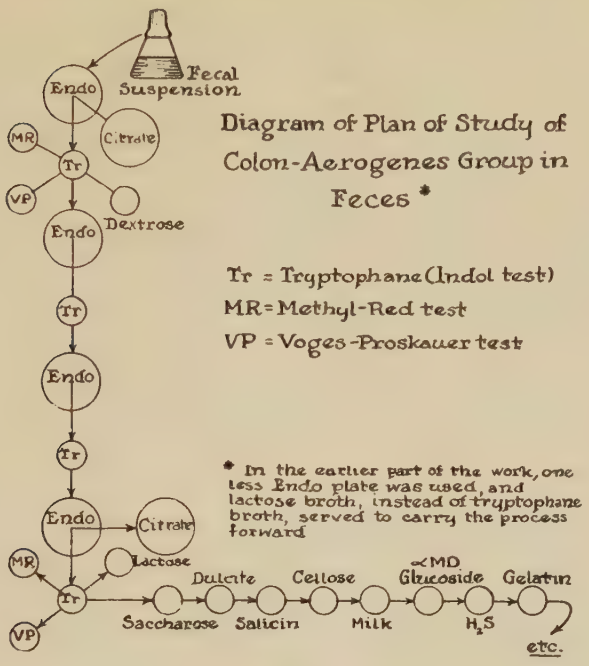

FIG. 1.

Strains failing to ultimately dissimulate lactose are dropped. Adequate sampling has been the rule, 11.2 organisms on average being picked from each of the 279 platings. The strains have been tested for their fermentation of dextrose, lactose, saccharose, dulcite, salicin, alpha-methyl-d-glucoside and cellose; for their methyl-red, Voges-Proskauer, citrate utilization, milk and gelatin reactions; and for the production of indol and hydrogen sulphide. Data pertinent to the topic of this paper are presented in Table I.*

The only group member found in several fresh fecal specimens was some form of Coli (Escherichia). Of the 1262 strains in the corrected summary only 2 were Intermediate (Citrobacter). On storage the 2-3% of Aerogenes (Aerobacter) originally present in fresh feces greatly increase and the Coli disappear—a process more marked for specimens ice box stored and in such specimens complete in about 5 weeks. Almost coincident with the disappearance of typical coli intermediates types appear and they increase and persist with cold storage. They occur also but are not prominent at any

* Intermediates are organisms of the Colon-Aerogenes group which possess one or more Coli characteristics and one or more Aerogenes properties. Aerogenes and Intermediates utilize citrate as a carbon source; Coli does not.

TABLE I.
Summary of all Data.

	Specimens examined	Times plated	Strains isolated	% strains Coli	% citrate utilizers Intermediates
Fresh feces	57	57	1454	94.6	30.7
37°C. storage	8	60	592	55.5	12.6
Ice box storage	12	162	1086	32.9	32.7
		Corrected Summary			
Fresh feces[2]	44	44	1262	97.7	7.1
37°C. storage	8	60	592	55.5	12.6
Ice box storage[3]	11	139	956	22.8[4]	32.7

[2] Corrected by omission of 13 platings from feces of babies less than 3 weeks old.

[3] Corrected by omission of 23 platings from specimen No. 4, in which the original feces contained so few citrate utilizers that only Coli were sampled.

[4] Were specimens stored at ice box temperature not plated before 5 weeks this figure would approach *zero*, where the original sample contained any citrate utilizers.

stage in the specimens held at 37°C. With further storage the *Aerogenes* become degraded, particularly with respect to lactose fermentation, and gelatin liquefiers appear. In early storage stages when Coli are disappearing indol negative Coli and Coli anaerogenous in all sugars appear. Slow lactose fermenting Coli have not been encountered under the conditions of this experiment. Practically all forms of the group which have been reported from soils, grains, waters, foodstuffs, etc., and not heretofore considered as fecal are to be found at one stage or another in stored feces. It is not true that the anomalous strains reported by many authors are necessarily mixed cultures.

On the other hand it is possible for a material quantity of feces stored many months to yield no form of the group other than Coli identical with those of fresh feces. Specimen 4, mentioned in the summary, illustrates this point, interpreted as due to the original fecal mass containing no viable citrate utilizing organisms. That such specimens do occur would seem to be evidence that distinct forms appear in feces at different times by virtue of succession rather than by variation. By contrast it seems most likely that the degraded forms are induced variations of species previously present. The feces of new-born babies on mother's milk contain so few Colon-Aerogenes organisms that they have not been found in the specimens examined even by enrichment of the material in lactose broth. In bottle-fed babies, by contrast, the Colon-Aerogenes flora is significant but it may be represented by so many citrate utilizing forms that the summary has been corrected for the 13 babies examined until a more thorough study of the group in babies can be made

to establish the significance of the Colon-Aerogenes distribution in this age group.

It will thus be seen that the question of which Colon-Aerogenes organisms are fecal and which are not is greatly complicated. Under certain circumstances feces may furnish such quantities of normal and degraded Aerogenes, gelatin liquefying Aerobacter or Cloacae, and Intermediates as to make untenable the assumption that such forms necessarily come from such environmental sources as soils and grains. Furthermore, the finding of Coli typical of those of freshly excreted feces may be no guarantee that the Coli found indicate recent fecal pollution.†

7772 P

Blood Pressure Response to a Standard Stimulus in the White and Negro Races.

EDWARD H. SCHWAB, DOLPH L. CURB,* J. L. MATTHEWS AND V. E. SCHULZE. (Introduced by George R. Herrmann.)

From the Department of the Practice of Medicine, Medical Branch, University of Texas, and the Cardiac Clinic of the John Sealy Hospital, Galveston, Texas.

A new concept of the etiology and pathogenesis of essential or arteriolar hypertension, based upon the neurogenic theory, has recently been brought forth by Hines and Brown.[1] According to them there exists a primary constitutional factor manifested by a hypersensitive or hyperreactive vasomotor system which is activated by secondary subsidiary factors in the form of environmental, infectious or toxic agents. Furthermore, they advanced the opinion that this constitutional abnormality should be capable of detection in early life, and, to attain this end, devised a standard vasomotor stimulus based upon the blood pressure response to the application of cold.

We have called attention[2] to our discovery of a rather striking difference in the incidence of hypertensive cardiovascular disease in the white and negro races in this locality. We, therefore, felt that, in view of this racial difference, an exceptional opportunity existed

† For much valuable assistance in this project we are indebted to Mrs. E. B. Crawford.

* Kass Scholarship Award Fellow.

1 Hines, E. A., Jr., and Brown, G. E., *Ann. Int. Med.*, 1933, **7**, 209.

2 Schwab, E. H., and Schulze, V. E., *Am. Heart J.*, 1932, **7**, 710.

to investigate further the possible neurogenic basis of essential hypertension. The "Cold Stimulation Test", with certain modifications, was applied to 325 young adults, 172 white patients and 153 negroes, approximately equally divided as to sex, and ranging in age from 15 to 35 years, with an average age of 24 years. All subjects were free from cardiovascular disease and had normal blood pressures. After a period of rest in the recumbent position, control blood pressure readings were made at frequent intervals until a constant basal level was reached. The hand and wrist of the opposite arm were then immersed in cold water (4 to 5°C.), and determinations were made after 30, 90 and 150 seconds had elapsed. The hand was then removed and readings were made until the blood pressure had returned to its previous resting level.

The results of the study are given in Table I. The cases are grouped according to the height to which the systolic blood pressure rose during the application of the stimulus. The average resting systolic blood pressure in the white subjects was 112 mm. of mercury and 115 mm. of mercury in the negroes. It is clearly shown that as the systolic blood pressure level is gradually raised, the percentage of negro cases attaining a given level slowly begins to exceed that

TABLE I.

Comparative Analysis of the Blood Pressure Response to a Standard Vasomotor Stimulus in the White and Negro Races.

Highest Systolic Blood Pressure Obtained During Application of the Stimulus mm. Hg. or above	White Subjects		Negro Subjects	
	No. Cases	%	No. Cases	%
100	172	100.0	153	100.0
105	170	98.8	153	100.0
110	169	98.2	153	100.0
115	165	95.9	149	97.3
120	156	90.6	144	94.0
125	138	80.2	128	83.6
130	109	63.3	112	73.1
135	92	53.5	93	60.7
140	70	40.7	75	49.0
145	48	27.9	60	39.2
150	34	19.8	43	28.1
155	18	10.5	27	17.6
160	7	4.1	20	13.1
165	5	2.9	17	11.1
170	1	0.6	8	5.2
175	0	0	7	4.6
180	0	0	5	3.3
185	0	0	4	2.6
190	0	0	3	2.0
195	0	0	2	1.3
200	0	0	1	0.7
205	0	0	0	0

of the white subjects and as the higher elevations are reached the percentage difference progressively becomes more marked.

From these observations we may conclude that there is a quantitative difference in the blood pressure reaction to a standard vasomotor stimulus in the white and negro races. This finding indicates that a hypersensitive vasomotor mechanism is more frequently encountered in the negro than in the white race. In view of the greater incidence of hypertensive cardiovascular disease in the negro race, these results seem to add support to the newer neurogenic concept of the development of the disease.

7773 P

Action of Ovarian Follicle Hormone in Ovarian Insufficiency in Women as Indicated by Vaginal Smears.*

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The treatment of women with ovarian insufficiency by ovarian follicular hormone (OFH) has fallen short of the expectations aroused by its successful use in animals. It is possible that the amounts of hormone necessary to produce comparable changes in man are so large[1] as to be out of the question except for occasional brief studies in humans.[2] On the other hand the failures may have been due to the lack of objective criteria for estimating dosage and evaluating effects; or the choice of ineffective modes of administration.

The aim of this study was the development of an objective method for evaluating OFH action in ovarian insufficiency. The study of vaginal smears has been of great value in observing the effects of OFH in rodents and other mammals. Recent studies[3] have demonstrated a cycle in the vaginal fluid of the human comparable to that in animals. The applicability of the vaginal smear method to the study of the effects of OFH in the human is briefly described in this report.

* This work has been aided by grants from the Committee for Research on Sex Problems, of the National Research Council, and the Council on Therapeutics of the American Medical Association.

1 Parkes, A. S., and Zuckerman, S., *J. Anat.*, 1931, **65**, 272.

2 Kaufmann, C., *Proc. Roy. Soc. Med.*, May, 1934.

3 Papanicolaou, G. N., *Am. J. Anat.*, 1933, **52**, supplement.

Fifteen women with ovarian insufficiency, following either bilateral ovariectomy or the menopause, were observed over periods of several weeks to a year. After a control period to establish the character of the vaginal smear and the absence of OFH in the urine, treatment was begun. Smears were taken regularly over long periods and at varying intervals after administration of the hormone. The dose of OFH was adjusted from time to time until changes occurred in the smear. Subjective symptoms, such as hot flushes, headaches, nervousness, mental depression, and libido were also noted.

Treatment was aimed at the production of the vaginal smear associated with the follicular (copulative) phase in the normal female.[3] This is characterized by leucopenia, and epithelial cells of the squamous type, largely cornified, with small pyknotic nuclei. It corresponds to the smear obtained in ovariectomized rodents with OFH. Vaginal smears of women who have been ovariectomized or are in the post-menopausal state are usually characteristic. There are many leucocytes and a predominance of either non-cornified squamous cells with larger nuclei, or of compact cells derived from the deeper layers of the vaginal epithelium, with large, well-preserved nuclei. These latter cells are rarely noted in the normal subject.

Preparations of OFH in oil for hypodermic use, and for oral administration, were chiefly employed in this study.†

Results. OFH in oil given subcutaneously produced specific changes in the vaginal fluid of all but one of fifteen women with ovarian insufficiency following bilateral ovariectomy or menopause. There was an increased vaginal secretion, and a gradual replacement of the epithelial cells characteristic of these conditions by squamous cells, largely cornified, with small pyknotic nuclei. A relative or absolute leucopenia often superseded the usual leucocytosis.

The amount of OFH in oil required to produce this effect by subcutaneous injection varied, in different patients, from 100 to 2000 rat units per day. The majority of patients responded to 500 RU/day. One failed to react to 2000 RU/day. OFH in oil was given by mouth to 6 of this same group of patients, in amounts of 2000 to 4000 RU/day. An increase in the vaginal secretion of mucus was often noted, but no specific cellular changes, except in one case receiving 4000 RU/day, in whose smear slight cellular alterations occurred. The effects of OFH were slightly cumulative, and disappeared within a few days after treatment was stopped.

† The ovarian follicular hormone preparations used in this study were kindly furnished by Dr. J. J. Durrett of E. R. Squibb and Sons.

With the changes in the smears there occurred subjective improvement, with diminution in the severity and number of hot flushes and headaches, lessened nervousness and depression, increased well-being, and occasionally increased libido.

7774 C

Effect of X-Ray on Experimental Encephalitis in Mice Inoculated with the St. Louis Strain.*

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Since perivascular lymphocytic infiltration is the essential lesion in encephalitis and since lymphocytes are highly sensitive to irradiation, it seemed logical that small doses of X-ray should be effective as a therapeutic measure. The treatment by X-ray of 3 cases of encephalitis in human patients has been reported.[1] Since then 4 other cases have been treated. One was an early case and recovered completely in a short time. Two others were more protracted, having had the disease for 3 months before treatment was instituted. In these 2 latter cases various groups of muscles were showing progressive paralysis. The patients showed marked improvement after a series of X-ray treatments and are symptom-free to date. The fourth case, one of 6 months' duration with early Parkensonian syndrome, is still under treatment but shows improvement.

Although the clinical evidence is very suggestive the findings are empirical and since the clinical studies can not be controlled, it was decided to carry out the treatments under experimental conditions. Mice are known to be highly susceptible to the St. Louis strain of virus.[2] The infective dose has been standardized and the incubation period is known.[3] If mice that were treated with X-ray after inoculation with the virus recovered or had the period of incubation

* X-ray exposures were under the supervision of Doctors C. F. Baker and W. J. Marquis in the Department of Radiology, Presbyterian Hospital, Newark, N. J.

1 Goldberg, S. A., Baker, C. F., and Hurff, J. W., *Radiology*, 1934, **22**, 663.

2 Webster, L. T., and Fite, G. L., *Science*, 1933, **78**, 463.

3 Brodie, M., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 1227.

or the duration of the disease prolonged, it seemed certain that the X-ray had a definite effect upon the disease.

A series of normal mice exposed to varying amounts of X-ray to determine how much they could tolerate, showed no apparent ill effects after a series of 10-minute treatments (each equivalent to ½ a human erythema dose) given daily for 10 days. Preliminary experiments showed that the small X-ray doses given 48 to 72 hours apart that proved so effective in human beings were not effective in mice because of the fulminating character of the disease. Therefore more prolonged treatments at 24-hour intervals were given. Each treatment was 135 K.V., 5 ma., distance 15 inches, filters 3 mm. aluminum, time 5 minutes, equivalent to one-quarter of a human skin erythema dose.

Two methods of inoculation with the encephalitis virus were employed. In the first, 10 mice inoculated by the intracerebral route were treated daily with X-ray. Untreated controls came down with symptoms on the third or fourth day after inoculation and died in 6 to 12 hours thereafter. Of the treated animals one died on the third day, 8 hours after symptoms appeared; 5 died on the fourth day, two 36 hours and three 12 hours after symptoms appeared; 4 died on the fifth day, one 54 hours and three 15 hours after symptoms appeared. While 3 of these ran a more prolonged course than the controls, the difference was not striking. Therefore this method of inoculation was abandoned in favor of the intranasal route, where treatments could be more extensively carried out.

Inoculated by the intranasal method, mice usually showed symptoms on the fifth to seventh day and died within 6 to 24 hours thereafter. Several series of mice were inoculated intranasally and given daily X-ray treatments until death or up to 12 treatments. The results were as follows: Of the 38 mice thus inoculated and treated 15 survived without any apparent symptoms, 4 survived, having shown symptoms on the sixth and seventh days, of the 19 that died the incubation period varied from 6 to 11 days with an average of 7.8 days; the duration of illness in these 19 was from 1 to 5 days with an average of 2.5 days; the number of days before death in this group varied from 6 to 11 days with an average of 9.5 days. The animals that were ill for several days were unable to take nourishment and their death may have been due to starvation. In all, 53 controls were used and the average day of survival was 6 days with symptoms for 6 to 24 hours. In this control group 8 died on the fifth day, 27 on the sixth, 11 on the seventh and 8 on the eighth day. All the controls died with the exception of one that recovered after a mild attack.

All of these mice received 0.03 cc. of a 1-10 dilution equivalent to about 10-50 infective doses, injected into one nostril. This was based on the following experiment which determined the dosage for intranasal inoculation. Four mice given 0.03 cc. of a 1-100 dilution died in 6 days. Two mice given 0.03 cc. of a 1-500 dilution died in 6 days. Seven mice were given 0.03 cc. of a 1-1000 dilution, 3 died and 4 survived after a mild attack.

Another group of 8 mice was inoculated with a double dose of virus, *i. e.*, 0.03 cc. of a 1-10 dilution in each nostril. Two controls died on the sixth day. In the treated group all showed symptoms on the sixth day. Three of these died on the seventh day, 2 on the eighth day and one on the ninth day.

TABLE I.

Effect in mice of repeated X-ray treatments begun 24-48 hours after intranasal insufflation of St. Louis strain of encephalitis virus.
Dose of virus 0.03 cc. of a 1-10 dilution.
Dose of X-ray equivalent to ¼ human erythema dose.

No. of Mice	No. X-ray Treatments	Incubation period days	Duration of illness days	Day of death	Survivors
8	0	5	1	5	0
27	0	6	1	6	0
11	0	7	1	7	0
6	0	8	1	8	0
1	0	6	3	0	1
1	4	6	1	6	0
1	5	6	3	8	0
1	6	6	3	8	0
2	7	6	3	8	0
1	7	6	3	9	0
1	6	8	2	9	0
1	6	7	3	9	0
1	6	6	4	9	0
2	7	8	3	10	0
1	8	8	3	10	0
1	6	7	5	11	0
2	7	10	2	11	0
2	8	10	2	11	0
1	9	11	1	11	0
1	9	10	2	11	0
9	10	0	0	0	9
6	11	0	0	0	6
1	11	6	3	0	1
3	12	6	2	0	3

Because of the small number of animals used no attempt has been made to determine how soon after inoculation X-ray treatments should be started to obtain optimum effects. Taking into consideration the extreme susceptibility of mice to this virus and the fulminating character of the disease, the results must be considered as important. Not only was the life span and the period of

incubation of the treated animals prolonged, but the duration of the disease with symptoms was prolonged to 2, 3, and 4 days. These data indicate that numerous X-ray treatments either prevent or cure encephalitis in a number of mice inoculated intranasally with the virus.

7775 P

Nerve Impulses from Receptors in the Cornea.

SARAH S. TOWER. (Introduced by D. W. Bronk.)

From the Eldridge Reeves Johnson Foundation for Medical Physics, the University of Pennsylvania.

The central portion of the human cornea yields, to any form of stimulation, only the sensation of pain. The reactions of laboratory mammals to stimulation of this field recapitulate those of the human. Therefore it may be inferred that the sensation evoked is likewise pain. The site thus offers the possibility of investigating the mechanism of this sense, unconfused by other modalities. To this end arrangements were made to amplify and record action potentials in the long ciliary nerves. In some experiments the active fibers of one of these nerves were reduced to 2 or 3. The animals, cats, were decerebrated, the second to sixth cranial nerves cut, and the ciliary ganglion removed. The stimulators employed included a set of von Frey needles (⅛ to 10 gm.), a corresponding set of hairs, a mechanical stimulator delivering prick of variable intensity, and blunt glass rods.

The responses obtained from the cornea using these different instruments were essentially the same—trains of impulses of rapidly diminishing frequency, or occasionally, from the extreme fringe of a fiber's field, a single impulse only. Initial frequency and duration of the discharge were functions, both, of the intensity of the stimulation. In the first hundredth-second frequencies exceeding 500 per second were obtained. Thereafter, with continued stimulation, the discharge fell off precipitately, sometimes to cease entirely in a few seconds, sometimes to establish a sustained activity at one to 5 impulses per second. Removing the stimulus then commonly caused a second small outburst. Not infrequently a single stimulation of the cornea caused 2 fibers to respond, one of which adapted completely, the other, not.

The single corneal ending was isolated, not by attack on the cornea, no part of which is supplied by a single fiber, but by attack on the nerves. One nerve fiber was then found distributed over roughly a quadrant of the cornea and extending onto the adjacent sclera. Yet within this large field were islands not supplied by the fiber in question, but perhaps, in a 2-fiber preparation, by the second of the survivors.

The question arises, what constitutes a nerve ending—each of the numerous small terminals on one nerve fiber, or the sum total of these? When mechanical stability was achieved, the trains of impulses were quite regular in a single fiber, making it unlikely that individual terminals were firing off independently. Yet stimulation anywhere except at the periphery of a fiber's distribution must deform many of these. Clearer evidence that the whole terminal ramification acts together, was given by another observation. From time to time preparations were obtained, in which an ending discharged spontaneously 2 to 4 times a second, for hours. Stimulation of the corneal terminals of such a "ticker" elicited the usual outburst of impulses, but following this the spontaneous firing slowed or ceased, sometimes for seconds. Stimulation anywhere within the extensive field of such a fiber produced this depression, and in proportion only to the amount of the discharge called forth. And excitation of other fibers within the area was quite without such effect. Moreover, the fatigue which the corneal endings showed as diminishing response in a series of stimulations was unequivocably shared by a "ticker". And similarly, spontaneous and excited activity suffered together in the progressive slow deterioration throughout the course of a long experiment.

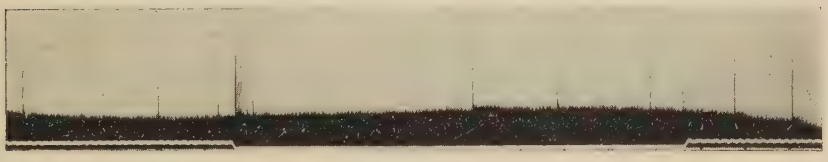

FIG. 1.

Action potentials in a 2-fiber preparation of a long ciliary nerve, one of the fibers of which was discharging spontaneously. The upper marker indicates the interval of application of a prick in the corneal field of this fiber. An initial discharge of impulse was followed by cessation of the spontaneous firing, which resumed later. The lower marker gives time in 1/5 sec.

On the other hand, within the terminal ramification local conditions determined both the initial frequency and the duration of the discharge resulting from a given stimulation. To begin with, all

parts of a fiber's distribution were not equally responsive. From point to point the discharge varied, but always with a general gradient of increasing activity from the periphery toward the center of the field. Again, the fatigue of repetitive stimulation of one point certainly acted most intensely at that point, as did the denial of access of aqueous humor to any portion of the cornea. Finally, adaptation was appreciable only at the point stimulated, for a second stimulus applied as closely as possible to a first to which adaptation was complete, resulted in a second vigorous discharge in the same nerve fiber.

Putting these facts together, the sensory ending in the cornea emerges as all the terminal tissue of one nerve fiber. This is a unit, activity in any part of which probably involves the whole. Moreover, there is no evidence that activity in this unit influences in any way the activity of closely associated units. Functionally, the total corneal sensory mechanism appears as an aggregate of units, and not as a continuum. No evidence has been forthcoming of the presence of more than one sensory mechanism in the cornea. By analogy with the human, this should be pain. More convincingly, the wide extent of the ending, and the spread of activity throughout it, reflect one of the characteristics of the pain sense as subjectively studied, its lack of any but the most general localization. Likewise, the initially rapid, but then often incomplete adaptation of the mechanism repeats another aspect of the same subjective experience. Therefore, perhaps, one may see in the properties of the sensory mechanism studied in the cornea, peripheral determinants of the central processes resulting in the sensation of pain.

7776 C

Oral Toxicity of Ortho-n-alkylphenols to White Rats.*

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In a search for an active non-toxic ascaricide which has been going on in this department for several years, several series of alkylphenols have been studied for their ascaricidal action as well as

* The funds for carrying out this work were given by the International Health Division of the Rockefeller Foundation.

their toxicity. It was found that in the 6-alkyl-meta-cresols[1] and the 4-n-alkyl-resorcinols[2] that the oral toxicity to rats decreased with increase in length of the alkyl radical. We wish to report the findings on the oral toxicity of ortho-n-alkylphenols. These substances were synthesized in this laboratory by Dr. R. W. Stoughton, Dr. R. Baltzly, and Mr. A. Bass.

The method of giving these substances to the rats has been described previously.[1] Five per cent ethylene glycol was added to phenol in order to liquefy it so that it might pass down the stomach tube. The alkyl-phenols are, however, liquids. The results of these toxicity studies are given in Fig. 1.

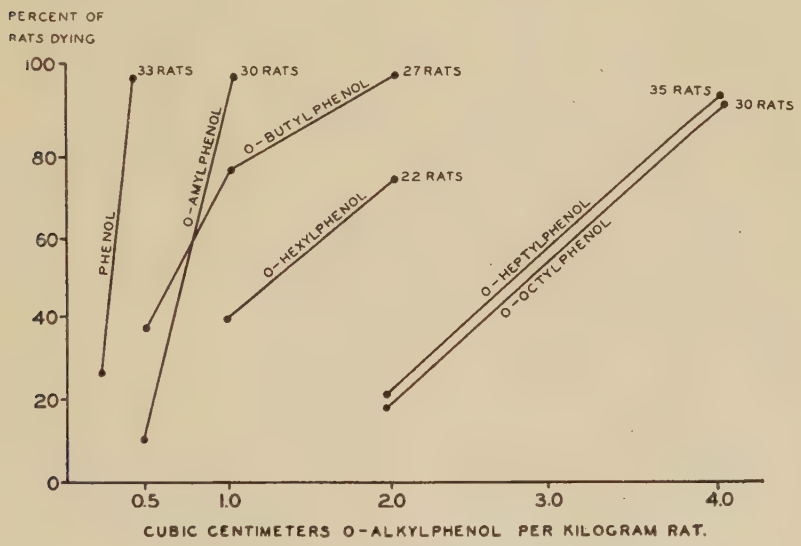

FIG. 1.

Toxicity of Ortho-alkylphenols to White Rats.

It will be seen that the toxicity of these substances decreases with increase in length of the alkyl radical. This is more strikingly shown in Table I, where the amounts of the different members of the series which resulted in the death of 50% of the rats are compared.

The members of these series of ortho-alkylphenols up to and including ortho-hexylphenol caused death rather quickly. Most of the rats died in from 2 to 6 hours while practically all of them were dead in from 8 to 12 hours. The ortho-heptylphenol and the ortho-

[1] Lamson and Brown, *J. Pharm. and Exp. Ther.*, in press.

[2] Lamson, Brown and Ward, *J. Pharm. and Exp. Therap.*, in press.

TABLE I.

Toxicity of 4-n-alkylresorcinols, ortho-alkylphenols and 6-alkyl-meta-cresols. Toxicity expressed as cubic centimeter of drug lethal to 50% of the rats.

resorcinol	0.25	phenol	0.30	m-cresol	0.35
				methyl-m-cresol	0.73
				ethyl-m-cresol	0.53
propylresorcinol	0.45			propyl-m-cresol	0.73
butylresorcinol	0.50	o-butylphenol	0.65	butyl-m-cresol	1.10
		o-amylphenol	0.70	amyl-m-cresol	1.50
hexylresorcinol	0.70	o-hexylphenol	1.30	hexyl-m-cresol	2.30
heptylresorcinol	1.30	o-heptylphenol	2.75	heptyl-m-cresol	3.32
		o-octylphenol	2.80	octyl-m-cresol	4.0
				nonyl-m-cresol	4.0

octylphenol acted more slowly and it was from 12 to 36 hours before those dosed with these substances died. At autopsy the rats usually showed considerable gastric enteritis and bloody fluid in the intestines. Convulsions were noted only in those rats given phenol. These began several minutes after dosing the rats and continued for several hours. That the decrease in toxicity of the higher alkylphenols is not entirely due to lack of absorption is shown by the fact that approximately 90% of a 2.0 cc. dose of n-ortho-heptylphenol is absorbed by both man and dog.

It will be seen in Table I that the alkylphenols of this series were appreciably less toxic than the alkylresorcinols with the corresponding number of carbons in the alkyl radical.

Conclusions. 1. The oral toxicity of ortho-n-alkylphenols to white rats decreases with the increase in length of the alkyl radical. 2. This decrease in toxicity with increase in length of the alkyl radical corresponds to our toxicity findings in series of 6-alkyl-meta-cresols and 4-n-alkylresorcinols. 3. Ortho-n-alkylphenols are less toxic than n-alkylresorcinols with the corresponding length of alkyl chains but show little difference in toxicity from the 6-alkyl-meta-cresols with the same number of substituted carbon atoms.

7777 P

Toxicity of Quinine, Quinidine, Hydroquinidine and Hydrocinchonidine in the Guinea Pig.

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The determinations were made in connection with observations on antimalarial value.[1, 2, 3] Guinea pigs, chiefly males, were injected subcutaneously in the flank with M/4 solutions in HCl to form very nearly the dihydrochlorides. Table I shows the numbers of animals dead or obviously dying 48 hours later, out of totals injected.

TABLE I. Toxicity of Four Cinchona Bases.

	Base	M.W.	Doses in mg.-mol. per kg. 0.3	0.4	0.5	0.6	0.7
1.	Quinine	324	0/4	0/8	8/30	14/30	22/30
1.	Quinidine	,,	7/30	17/30	26/30	7/8	—
2.	Quinine	,,	—	—	—	19/30	—
2.	Hydroquinidine	326	—	13/30	—	—	—
3.	Hydrocinchonidine	296	—	—	—	—	15/30

The quinidine contained 6.7% hydroquinidine,[4] a customary impurity, to as high as 30%,[4] in quinidine from cinchona bark but not in that from cuprea bark.[5] These 2 bases seem not to differ much in toxicity. Series 1 is a composite of observations on 8 groups of animals injected on as many different occasions, on each of which, except once, some received quinine and some quinidine. The error for composite results is usually small[6] so that we probably obtain an approximation to the 50% lethal dose (LD 50)[7] and the LD 25 and LD 75. These once obtained for quinine and quinidine, approximate LD 50s were predicted from previous rather slight data on the other bases and proved substantially correct, as shown. No animal was injected on more than one occasion.

* This work has been aided by a grant from the Committee on Scientific Research of the American Medical Association.

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2 Bevil, H. G., *Am. J. Trop. Med.*, 1932, **12**, 473.

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5 Leger, E., *Les alcaloides des quinquinas*, 1896.

6 Gaddum, J. H., Medical Research Council, Special Report Series, No. 183, 1933.

7 Trevan, J. W., *Proc. Roy. Soc. B.*, 1927, **101**, 483.

7778 P

Influence of Site of Subcutaneous Injection upon Toxicity Figures.

W. T. DAWSON.*

From the Department of Pharmacology of the University of Texas School of Medicine, Galveston, Texas.

Cupreine (M.W. 310) and quinine, its methyl ether[1] (M.W. 324), were injected subcutaneously in the flank in guinea pigs, chiefly males, in M/4 solutions in HCl to form very nearly the dihydrochlorides. Table I, Series 1, shows the results, deaths with-

TABLE I.
Influence of Site of Subcutaneous Injection upon Toxicity Figures of Cupreine and Quinine.

	Date	Doses in mg.-mol. per kg. 0.4		0.6		0.8	
		Series 1. Flank Injections					
Quinine	Feb. 22	1/10	(1/10)	5/10	(7/10)	7/10	(8/10)
	Mar. 8	0/10	(1/10)	1/10	(2/10)	7/10	(8/10)
	Mar. 16	0/10	(0/10)	—	—	—	—
	Totals	1/30	(2/30)	6/20	(9/20)	14/20	(16/20)
Cupreine	Feb. 22	2/10	(4/10)	6/10	(8/10)	4/10	(10/10)
	Mar. 8	0/10	(3/10)	0/10	(1/10)	3/10	(7/10)
	Mar. 16	2/10	(3/10)	—	—	—	—
	Totals	4/30	(10/30)	6/20	(9/20)	7/20	(17/20)
		Series 2. Dorsal Injections.					
Quinine	Mar. 16	—	—	—	—	9/10	(9/10)
Cupreine	Mar. 16	—	—	—	—	0/10	(0/10)
		0.5	0.75	1.00	1.25	1.50	1.75	2.00
Quinine	Various	5/56	19/36	50/57 16/19†	—	—	—	—
Cupreine	Various	—	—	—	0/5 (0/5)	0/5 (0/5)	1/5 (1/5)	2/11 7/19†

† Animals previously injected with cinchona bases and recovered.

in 48 hours first, those within 7 days next and in brackets. The 48-hour toxicity curves intersect, although an earlier report[1] assigned to cupreine a toxicity to the guinea pig half that of quinine. With cupreine great irregularity is here seen and late deaths are frequent. Necropsies showed evidence of passage of both bases from the flank site toward or into the abdominal cavity, internal hemorrhage, mesenteric vascular engorgement, intestinal adhesions and necrosis all

*This work has been aided by a grant from the Committee on Scientific Research of the American Medical Association.

[1] Grimaux, E., Laborde and Bourru, *Compt. rend. acad. des sciences*, 1894, **118**, 1303. Von Oettingen, W. F., Therapeutic Agents of the Quinoline Group, Chemical Catalog Co., 1933.

being noted. It was thought that cupreine might penetrate tissue and reach the peritoneal cavity more readily than quinine, and undergo in this experiment more frequently an accelerated absorption. Later therefore (Series 1 and 2) the site of injection was shifted for half the animals to the dorsum so as to hinder penetration into the abdominal cavity. The toxicity of the cupreine plainly fell (March 16), that of quinine remained of the same order as before. Late deaths became negligible. Further observations (Series 2) indicate that this shift of injection site makes little difference in the 50% lethal dose of quinine but probably doubles that of cupreine. It is clear that in comparative toxicity determinations the use of relatively large numbers of animals on each dose may by itself be inadequate to give reliable results; the method must be given close scrutiny.

7779 P

Creatine Content of Heart in Experimental Cardiac Hypertrophy Due to Hyperthyroidism.

M. BODANSKY AND J. F. PILCHER.

From the Departments of Pathological Chemistry and Pathology, University of Texas Medical School, and the Laboratories of the John Sealy Hospital, Galveston.

Cowan[1] has recently reported that the administration of thyroxin to rats, while producing an increase in ventricular muscle mass, resulted in a loss of creatine. Before the publication of Cowan's data, we had independently observed the same phenomenon in rats receiving desiccated thyroid. Indeed, the creatine values were depressed to much lower levels in our experiments than in those of Cowan. While it is to be recognized that the response to thyroid varies somewhat in different individuals, and depends upon a variety of factors to be described later, our results have been averaged as outlined in Table I for the purpose of comparison with Cowan's data. The usual daily dose of the powdered thyroid was 200 to 250 mg. per 100 gm. of body weight.

That the increased size of the heart represents a true hypertrophy is indicated by the fact that the water content of the hyperthyroid ventricles was practically the same as that of the normal ventricles (average of 76.48% for 11 hyperthyroid hearts and 76.21% for 6 normal hearts). On histological examination the hearts of the

[1] Cowan, D. W., *Am. J. Physiol.*, 1934, **109**, 312.

TABLE I.
Average Data Obtained in a Series of 27 Hyperthyroid and 8 Normal Control Rats.

	Normals	Hyperthyroid
Initial body wt., gm.		224
Final body wt., gm.	197	178
Wt. of ventricles, mg.	510.4	768.8
Ventricular wt./body wt. ratio	2.59	(3.43)* 4.32
Creatine concentration in ventricles, mg. %	198.3	104.4
Total creatine in ventricles, mg.	.996	.825

* The upper figure is calculated on the basis of original body weight; the lower on the basis of the final weight.

hyperthyroid rats showed hypertrophy, signs of acute degeneration, mononuclear cellular infiltration, and occasionally fatty metamorphosis.

Not infrequently death overtook the thyrotoxic animals in a sitting posture, sometimes following relatively slight exertion, and without being preceded by the usual signs of serious illness.

Our acknowledgment is due to Eli Lilly and Company for their generous cooperation in supplying a part of the desiccated thyroid used in these experiments.

7780 C

Differential Effect of Some Gonadotropic Substances on Development of Cyclical Sex Characters in the English Sparrow.*

EMIL WITSCHI AND W. N. KECK.

From the State University of Iowa.

The English Sparrow, like all other wild birds of the temperate zone, has a cyclical sexuality. During the breeding season in spring and early summer the sex glands are large and active; in July or August they undergo a rapid involution. In the males the testes shrink to approximately one-thousandth of their maximal size and the ovary of the females regresses to about the condition characteristic of immature birds 2 or 3 months old. Some of the secondary sex characters, especially bill color and gonadal ducts, follow this seasonal cycle. During the period of gonadal involution the bill of the male is as light as that of a castrate and the oviduct of the female is as thin and straight as that of an immature or an ovariotomized

* This investigation was supported by grants from the Committee for Research in Problems of Sex of the National Research Council.

bird. On the other hand during the breeding season the male bill is jet black and the female oviduct is voluminous and convoluted. Keck[1] has shown that these secondary sex characters are directly controlled by the corresponding male and female sex hormones. It is evident, therefore, that the involuted gonads of fall sparrows release sex hormones either not at all or in subthreshold quantities.

In an attempt to determine the possible rôle played by the hypophysis in this seasonal sex cycle, gonadotropic substances were injected into sparrows in sexual inactivity. In the winter of 1932 a group of quiescent males was injected with extracts from pregnancy urine, prepared and standardized in rat units in our own laboratory. No response was obtained, even though the exorbitant dosage of 50 units was administered daily during the whole month to some of the birds (Table I). Success came, however, when extracts of the anterior

TABLE I.
Male sparrows in quiescent phase injected with gonadotropic extracts.

Source of hormone	Daily amt. in rat units	No. injections	No. animals	Response
Horse Hyp. (Hill 4)	2	33	1	+
" " "	2	13	1	+
" " "	2	18	1	+
" " "	2	3	1	+
" " "	2	5	1	+
" " "	2	7	2	+2
" " "	2	17	1	+
Beef Hyp. (Kamm A.)	1	24	2	+2
" " "	1	20	1	+
" " "	2.5	7	1	+
" " "	2.5	17	1	+
Pregnancy Urine	1	30	4	—4
" "	10	30	4	—4
" "	25	30	4	—4
" "	50	30	3	—3
Pregnancy Urine (Kamm A.S.)	10	18	3	—3
" " " "	20	23	1	(+)

lobe of horse pituitary (preparation R. T. Hill) were given. As early as 4 hours after the third injection (52 hours after the first) the testes are considerably enlarged. The seminal tubules are inflated; their diameter is 1.5 times that maintained in the quiescent phase. Spermatogonial mitoses, never observed in the controls, are abundant. At the end of one week, primary spermatocytes make their appearance. During the third week, secondary spermatocytes and spermatids are added and in a male injected for 33 days even mature spermatozoa are present. The growth of the testes is shown in Fig. 1. At 17 to 18 days testes of 2 males have increased their

[1] Keck, W. N., *J. Exp. Zool.*, 1934, **67**, 315.

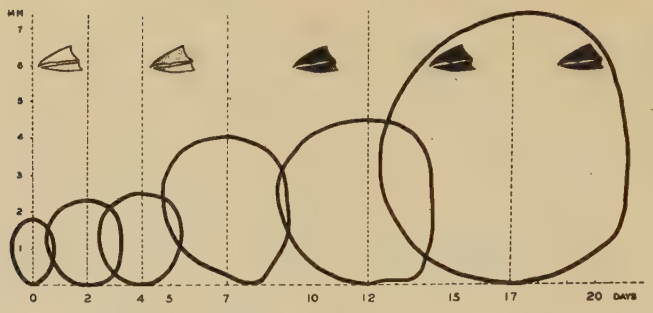

FIG. 1.

Effects of daily injections of 2 rat units of "follicle stimulating" hormone extracted from anterior lobe of hypophysis on testicular size and bill pigment. (The bills are not drawn to scale.)

volume by 100 to 150 times. The testes of the male with 33 injections (not represented in Fig. 1) are about 210 times as large as those of the controls. As one would expect, the growing testes liberate increasing amounts of male sex hormone which presently are recorded by the changing bill color (Fig. 1). On the fifth day a purplish tint is first noticeable which soon turns to blue, due to the accumulation of black pigment in the deeper strata of the horny bill. As time passes, this pigment is moved toward the surface and the bill appears darker and darker. The deepest jet black is not acquired before the end of the third week. These striking effects were obtained with daily injections of about 2 rat units only (Table I). Recently we received through the courtesy of Dr. Oliver Kamm 2 products from the Research Laboratories of Parke, Davis and Company, which made it possible to repeat our experiments (Table I). The first ("Antuitrin") is an extract of beef anterior lobe, containing mainly the follicle stimulating fraction of the gonadotropic hormones. Three males were injected with 1 rat unit daily. At the end of 20-24 days the testes show an increase to about 60 times the volume of controls. Two males injected with 2.5 rat units daily for shorter periods show about the same rate of development as those of our first series. The second product ("Antuitrin S") was extracted from pregnancy urine. Three males injected during 18 days with 10 rat units daily do not show even a trace of growth. However, the male that received 20 units during a period of 23 days had moderately enlarged testes (20 times the volume of controls). This raises the question whether we deal with an irregular spontaneous activation or with a "synergetic" effect (Evans, *et al.*[2, 3]). It can

[2] Evans, H. M., Meyer, K., and Simpson, M. E., PROC. SOC. EXP. BIOL. AND MED., 1931, **28**, 845.

[3] Evans, H. M., Pencharz, R. I., and Simpson, M. E., *Endocrinology*, 1934, **18**, 601 and 607.

safely be assumed that this male, which had been newly caught, was slightly stimulated by recent sunny weather. However, since the bird was kept in captivity this alone should have remained without consequences—as it did in a large number of controls caught on the same day. Further experiments will have to prove whether the combination of a subthreshold dose of hypophysis hormone with the otherwise inactive principle of pregnancy urine can stimulate testicular development in the sparrow. It is of interest that in the present case the thyroid is not enlarged (see below).

Experiments with females give equally striking results (Table II). Fig. 2 represents the effect of 16 daily injections of 2 rat units

TABLE II.
Female sparrows in quiescent phase injected with gonadotropic extracts.

Source of hormone	Daily amt. in rat units	No. injections	No. animals	Response	Weight mg.
Horse hyp. (Hill 4)	2	33	1	+	140*
" " "	2	16	1	+	**
Beef hyp. (Kamm A)	1	24	3	+1 (?)2	58.6; 10; 9.4
" " "	2.5	24	1	+	700
Preg. urine (Kamm A.S.)	10	18	2	—2	6.8; 1.6
" " " "	10	20	1	—	10
" " " "	20	23	1	—	6.1

* Estimated 1000 mg. at biopsy on 22nd day. ** Estimated 500 mg.; fig. 2 (right).

FIG. 2.
Effects of 16 daily injections of 2 rat units of "follicle stimulating" hormone extracted from anterior lobe of hypophysis on the development of the ovary and the oviduct of the English Sparrow. Left: largest of controls. Right: injected bird. × 2.

of Hill's extract. The ovary has attained about 50 times the weight of controls. A larger number of eggs than ever seen in normal lay-

ing females have entered the final growth phase. The oviduct shows an equally high development, indicating the production of female sex hormone by the ovary. A second female which received the same treatment was biopsied on the 22nd day. Its ovary was found about twice as large. Some of its largest follicles were punctured at that occasion. Injections were discontinued on the 33rd day. When on the 35th day the bird was killed its ovary contained at least a hundred middle-sized eggs (diameter 1-2 mm.), while the largest ones had all disappeared. The oviduct was still of maximal size. Administration of 2.5 units of Dr. Kamm's beef-antuitrin gave a similar result. On the 24th day the ovary had 70 times the weight of the heaviest of controls. Ten eggs had diameters of from 4 to 6 mm. Injections of 1 rat unit daily give only inconstant threshold reactions. Of three females treated with this dosage one shows a good response. Its ovary has 6 times the weight of the best controls and the largest egg measures 4 mm. The oviduct shows a very marked though not the maximal development. The 2 other females have ovaries not heavier than those of the heaviest controls. Their thyroids, however, are very much enlarged, like those of all males and females injected with hypophysis extracts (more than 3 times the volume of those from birds injected with urine preparations). Four females injected with relatively high doses of Antuitrin S do not show growth of ovaries or thyroids.

The failure of pregnancy urine (prolan or Antuitrin S) to stimulate the quiescent and involuted gonads of the sparrow is in harmony with the statement by Riddle and Polhemus[4] and by Schockaert[5] of its ineffectiveness to bring about precocious development of sex organs in immature doves, pigeons, ducks and chicks. The question arises, therefore, whether the bird differs from the mammal by the responsiveness of its gonads or by the failure of "synergetic" action of the hypophysis. No final answer can be offered yet, though a comparison with conditions in hypophysectomized rats suggests fundamental differences in the mechanism of gonadal reactions. Starting with a preliminary note for Evans' laboratory[6] a number of excellent studies, mainly by Collip, Smith, Evans and their respective coworkers, have recently come forth which disclose that the ovaries and testes of hypophysectomized rats are slow to react on pregnancy urine injections. With relatively high doses

[4] Riddle, O., and Polhemus, I., *Am. J. Physiol.*, 1931, **98**, 121.

[5] Schockaert, J. A., *Am. J. Physiol.*, 1933, **105**, 497.

[6] Reichert, F. L., Pencharz, R. I., Simpson, M. E., Meyer, K., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1931, **28**, 843.

the endocrine system and consequently the development of secondary sex characters can be stimulated. Follicular growth seems never to follow and spermatogenesis is only incompletely restored. It is evident that the gonads of birds are even less responsive to pregnancy urine extracts than those of hypophysectomized rats. At the present it seems that this principle is entirely foreign to and not utilizable by the bird.

A most characteristic feature of the endocrinology of the seasonal cycles of the sparrow is the near coincidence of minima and maxima in hypophyseal and gonadal activity. Our experiments indicate that gonadal development is stimulated by increased hypophyseal activity; but on the other hand, gonadal involution and cessation of the production of sex hormones do not incite renewed production of gonadotropic hormones. The sexual cycle seems to be entirely and one-sidedly directed by seasonal changes in hypophyseal functions.

7781 C

Origin of Functional Differences Between Male and Female Hypophyses.*

CARROLL A. PFEIFFER. (Introduced by E. Witschi.)

From the Zoological Laboratory, State University of Iowa.

Recent investigations have shown that there is a functional, and even an histological difference between male and female hypophyses. This presupposes either (1) a primary difference which is genetical and determined by the sex genes, or, (2) a secondary difference which is determined by the gonad function and is, therefore, dependent upon whether an ovary or a testis is present.

In order to investigate these two possibilities, testes of new-born male rats were transplanted into littermate females and the resulting disturbance in the endocrine system followed by the vaginal smear method. Since in the newborn rat the heat regulatory mechanism has not been established, animals can be operated after being rendered insensible and immotile by cooling on ice. Out of 150 females raised to puberty, 56 had the grafts resorbed; 59 established normal oestrus cycles even though grafts persisted; however, 35 females, all with well-growing grafts, gave evidence of an altered

* Aided by a grant from the Committee for Research in Problems of Sex of the National Research Council; grant administered by Prof. Emil Witschi.

endocrine function. The grafts were placed in various locations, but only those in the throat region will be further discussed here. In a later paper we intend also to report on the transplantation of ovaries of the newborn into newborn males and females and of gonads of the newborn into adults.

Of 48 females which had testes from litter-mate males grafted into the throat region, 10 were autopsied before puberty to serve for study of the development of graft and host gonads. Of the remaining 38, 30 carried growing grafts at the end of 7 to 9 months. Constant oestrus developed in 12 female hosts, 9 starting at puberty and 3 after various periods of irregular cycles. Of the animals which ran normal cycles, 6 were bred, and raised normal litters, which adds proof that these females are normal despite the presence of testis grafts. On the other hand, constant oestrus animals do not copulate unless, as may occur in rare instances, the constant oestrus is broken for a day or so. The animals may then copulate at the end of the nucleated epithelium stage but do not become pregnant.

Histological study demonstrates that this "constant oestrus" is in fact a suboestral condition. The ovaries contain follicles which in many cases are in excess of the number found in the normal ones. However, at about the time the follicles reach mature size the ova fragment and degenerate. Corpora lutea are not produced. The uterus remains in a quiescent condition and does not become distended with fluid as in normal oestrus or during the constant oestrus of a female in parabiosis with a castrate (Martins,[1] Witschi and Levine[2]). These conditions resemble those found in the case of an immature female rat responding to a minimal dose of follicle stimulating hormone. Therefore, this suboestral condition seems to indicate a constant low rate of production of follicle stimulating hormone by the hypophysis. Microscopically the hypophyses are normal, though cell counts were not made, nor was it attempted to decide whether the histological picture resembled more the male or the female type.

The grafts vary rather markedly in the type of tissues persisting. The germ cells disappear first, the tubules next, and the epididymis last. It is significant that in all cases of constant oestrus, testis tubules with numerous germ cells are present in the graft. However, spermatogenesis seems never to proceed beyond the secondary

[1] Martins, Th., *Endocrinol.*, 1931, **15**, 421.

[2] Witschi, E., and Levine, W. T., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 101.

spermatocyte stage, where degeneration takes place. At any rate the implants maintain a more nearly normal condition in the "constant oestrus" than in the "normal oestrus" group. It is a surprising fact that without exception the removal of the graft does not alter the endocrine disturbance once established. The animal remains in constant oestrus until autopsy (which in some cases was 9 months after removal of the graft). In one case constant oestrus developed even though the testis graft was removed shortly *before* puberty.

Injection of large doses of oestrin decreases the output of follicle stimulator as evidenced by cessation of follicular development. The larger follicles disappear, and if the injections are discontinued, the animals go into dioestrus for a while then return to "constant oestrus" without the formation of corpora lutea. However, large doses of luteinizing hormone cause the formation of corpora lutea and a dioestrus condition of the vagina in 5 days. These facts show that the hypophysis of the "constant oestrus" animal has ceased producing luteinizing hormone.

Goodman[3] has described among other experiments the implantation of the ovary into the eye chamber of the adult male rat. Under the influence of the male hypophysis the ovary shows follicular development but no luteinization unless injected with antuitrin S which contains predominantly luteinizing hormone. This report is of great value as the first detailed description of the development of the ovary under control of a male hypophysis. The identical behavior of ovaries in Goodman's experiment and in our "constant oestrus" group is evident and supports the conclusion that in our experiment the hypophysis has been reversed to the male type.

Summary. Transplantation of testes of newborn males into the neck region of littermate females gives "takes" in 80% of the cases. In 48% the grafts do not interfere with normal endocrine or reproductive functions of the carrier, while in 32% the implanted testis assumes control of the sexual differentiation of the hypophysis. The male type hypophysis stimulates follicular development but not luteinization in the host ovary. These hosts start constant oestrus and maintain this condition after the removal of the testicular graft. It is concluded (1) that the cases with "constant oestrus" are those in which the implanted testis is sufficiently active to have a leading influence upon the hypophysis which responds with the male type of constant non-cyclic function; (2) that the hypophysis is permanently altered; (3) that the sex type of the hypophysis is secondary, depending upon the presence of differentiated sex glands.

[3] Goodman, L., *Anat. Rec.*, 1934, **59**, 223.

7782 C

A New Method for the Preparation of Thrombin.

ALICE C. ROBERTS. (Introduced by William H. Howell.)

From the Laboratories of Physiological Hygiene, Johns Hopkins University, and from the Physiology Laboratory, George Washington University.

In the preparation of thrombin (or prothrombin) 4 different methods have been followed. The method of Schmidt[1] starts with fresh serum which is precipitated by a large excess of alcohol, the precipitate subsequently being extracted with water or saline solution. The method of Buchanan as modified subsequently by Gamgee[2] and by Howell[3] starts with the fibrin of the clot from which the thrombin is extracted by a strong saline solution and purified by acetone precipitation. The method of Mellanby,[4] as modified by Bleibtreu,[5] and by Mellanby,[6] starts with oxalated plasma, from which, after strong dilution, the fibrinogen and prothrombin are precipitated together by the addition of weak acid. The separation of the prothrombin from the fibrinogen is effected in different ways. The method of Bordet[7] also starts with oxalated plasma from which the prothrombin (serozyme) is precipitated by adsorption upon a suspension of tricalcium phosphate, or, as suggested by Fuchs, by adsorption upon magnesium hydroxide. It is well known that the original method of Schmidt usually gives a feeble preparation of thrombin which is not wholly satisfactory for purposes of experiment or demonstration. The methods described by Howell, Mellanby, Bleibtreu and Bordet, on the other hand, give, under favorable conditions, very potent preparations which cause rapid and strong coagulation of oxalated plasma or fibrinogen solutions. Experience in this laboratory has shown that the method of Howell does not always give a satisfactory product. With some specimens of fibrin, the yield is excellent, while with others it is poor. The principal object of the work here reported was to restudy and improve this method so as to obtain a more uniform preparation of thrombin and

[1] Schmidt, Alexander, *Zur Blutlehre*, F. C. W. Vogel, Leipzig, 1892.

[2] Gamgee, A., *J. Physiol.*, 1879, **2**, 145; Physiological Chemistry, 1880, Macmillan and Co., London.

[3] Howell, W. H., *Am. J. Physiol.*, 1913, **32**, 264.

[4] Mellanby, J., *J. Physiol.*, 1909, **38**, 28.

[5] Bleibtreu, M., *Pflüger's Arch.*, 1929, **213**, 642.

[6] Mellanby, J., *Proc. Roy. Soc. B.*, 1930, **107**, 271; 1933, **113**, 93.

[7] Bordet, J., et Delange, L., *Bull. Soc. Roy. Sc. Méd. et Nat. Bruxelles*, 1914, **72**, 87.

if possible a preparation free from admixture with impurities. The attempt to improve the method has proved to be a more difficult task than was anticipated. After many unsuccessful attempts, modifications have been developed which it is believed, are actual improvements and insure a good yield of very active thrombin in a state of considerable purity. In the absence of definite knowledge of the chemical nature of thrombin it is not possible to establish a wholly satisfactory criterion of its purity. The criterion that has been used in this work is the absence of any detectable amount of heat coagulable protein in the solutions of the thrombin as finally obtained.

As a basis for this work Howell's second method for the preparation of thrombin was followed.

Three principal changes were made in this method of which the first was the choice of fibrin. Experience with the blood of the different slaughter house animals that could be obtained readily for such work, pig, ox, and calf, showed that fibrin from very young calves gives by far the best yield of thrombin. The second change was the omission of the precipitation of the saline extract of the fibrin by acetone since this procedure appeared to inactivate a part of the thrombin, subsequent extracts being less effective or less potent than the original saline extract. In place of the method of precipitation by acetone as a means of removing other proteins there was substituted the method of prolonged dialysis against a buffer mixture of phosphates as described in detail below. The third change was the preliminary extraction of the washed fibrin with ether for 24 hours or more prior to the extraction of thrombin by strong saline. The resulting saline extract was less syrupy in character and contained less foreign matter than when the ether extraction was omitted.

Fresh fibrin from whipped calves blood is washed free of hemoglobin by squeezing it repeatedly in cold, running water. This material is then run through a food chopper to free it further from any trace of hemoglobin and to place it in suitable form for extraction. It is now placed in ether at room temperature and allowed to remain at least 24 hours or more in a closed container, or until it is ready for use. At that time the ether is filtered off, the fibrin washed with clean ether and finally dried before an electric fan to the point where the odor of ether disappears. It is now extracted with a volume of 8% sodium chloride equal to its own volume, for 12 hours in the ice chest. The extract is filtered off through 2 thicknesses of filter paper.

This filtrate is then dialyzed in cellophane tubing for several hours against distilled water to remove the excess of sodium chloride. It is advisable at this point to test the thrombic action of the extract upon some oxalated plasma to ascertain whether a sufficiently active preparation has been obtained. The solution is next dialyzed for 72 hours against a Sørensen buffer mixture pH 7.38 (95 gm. Na_2HPO_4 and 19 gm. KH_2PO_4 in 20 liters of water). During this period the dialyzing liquid is renewed either by a continuous slow stream or by changing the outside liquid half a dozen or more times. The material is finally dialyzed for 5 hours against running, distilled water to remove the phosphates. The solution is filtered without pressure through a No. 5 Whatman filter paper and the now clear filtrate is dried at room temperature before an electric fan. The material thus obtained is soluble in water and the solutions show strong thrombic action. Two drops of a 0.7% solution will cause clotting of 3 drops of oxalated plasma in approximately 30 seconds with most specimens. In dry form the potency of the preparation is retained apparently indefinitely. Saline solutions give neither precipitate nor opalescence on boiling, indicating the absence of heat coagulable protein. Positive reactions for protein are given by the biuret, xanthoproteic, Adamkiewicz, ninhydrin, and Folin-Denis reagents. Positive reactions are given also for the presence of sulfur (cystine) and phosphorus. Tests with the Molisch reagent and for purine bases were negative. Prolonged dialysis of the solutions against distilled water inactivates this thrombin indicating, possibly, that it is a protein of the globulin group.

7783 C

Number of Thrombocytes and Leucocytes in Blood of Adrenalectomized Rats.

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From the Department of Physiology, The Ohio State University.

Peripheral blood was obtained from each of a group of 15 normal rats for determining the total number of thrombocytes. Each blood sample was collected in a Trenner automatic red cell pipette and diluted one part to 200 with Ringer's solution (Casey and Helmer) and a small amount of cresyl blue added. After being shaken for 5 minutes in an automatic shaker, the platelets in 240 small squares

TABLE I.
Effect of Adrenalectomy on Platelet Count.

Animal No.	Before Adrenalectomy			Days after Adrenalectomy				Days to death
	1st Count	2nd Count	Nor. Ave.	2	4	6	8	
M 1	696,000	597,000	641,500	480,000	506,000	976,250	1,305,000	11
M 2	558,500	586,000	565,500	518,000	555,000	1,021,500	Death in 8 days	
M 3	602,500	604,500	603,500	499,000	501,000	1,040,000	1,055,000	11
M 4	493,500	472,500	483,000	353,000	410,000	Death in 7 days		
M 5	482,000	510,000	496,000	467,000	760,500	Death in 5 days		
M 6	606,500	554,000	580,250	570,000	705,000	Death in 5 days		
F 7	258,500	365,000	311,750	506,000	525,000	504,750	682,500	11
F 8	295,000	399,750	347,370	612,500	765,000	Death in 6 days		
F 9	234,000	230,000	232,000	364,000	447,000	838,500	Death in 9 days	
M 10	523,500	541,000	532,250	440,500	597,000	918,000	802,000	12
M 11	659,000	472,000	565,000	589,000	831,250	1,126,500	842,500	10
M 12	469,000	452,500	466,750	472,500	644,000	713,000	690,500	14
M 13	363,000	397,750	380,750	373,500	550,000	853,000		7

M—Male F—Female

on both sides of a double chambered Neubauer hemocytometer were counted by 2 workers for each animal.

After the average normal platelet count was obtained, the rat was bilaterally adrenalectomized. Two days were allowed for recovery from the operation and on alternate days, thereafter, the numbers of platelets were enumerated until death occurred from adrenal insufficiency. The results obtained on the 13 rats of the group which died of adrenal insufficiency are recorded in Table I. They averaged 477,350 in the normal state and 840,270 just before death, an average increase of 76%.

The total number of white cells in the blood of 34 normal rats averaged 11,319 while just before death from adrenal insufficiency the leucocytes averaged 17,576 per cubic millimeter of blood. This leucocytosis is in agreement with the observations made by Zwemer and Lyons[1] and also by Corey and Britton[2] on adrenalectomized cats.

Differential white cell counts were made on a group of 19 rats before and after adrenalectomy. In the normal state the lymphocytes averaged 81.2%, two days after operation 84.7% and just before death from adrenal insufficiency 88.4%. With the increase in lymphocytes there was a concomitant decrease in the neutrophils. The numbers of the other types of white blood cells seemed unchanged.

7784 P

Bacteriostatic Action of Irradiated Dye Media

HAROLD LEON FRUITMAN. (Introduced by H. F. Blum.)

From the Laboratories of the San Francisco Water Department of the City and County of San Francisco, California.

The writer has observed that the growth of bacterial organisms is inhibited on culture plates which contain photosensitizing dyes, and which have been exposed to light of relatively low intensities. The culture media used were eosin-methylene blue, and Salle plates, the latter containing erythrosine, brom-cresol-purple, and methylene blue. When *B. coli* and *B. aerogenes* were streaked upon such irradiated plates, growth generally did not occur when the plates were subsequently incubated at 37°C. Plain agar plates containing no

[1] Zwemer, R. L., and Lyons, G., *Am. J. Physiol.*, 1928, **96**, 545.

[2] Corey, E. L., and Britton, S. W., *Am. J. Physiol.*, 1932, **102**, 699.

dyes showed no inhibition of growth when subjected to the same treatment.

In some cases the quantity of light which produced these changes in the plates was very small, *e. g.*, an exposure 2½ minutes to the diffuse light of the laboratory inhibited growth on the Salle plates, although 30 minutes was the minimum time of exposure which produced inhibition on eosin-methylene blue.

Measurements of the intensity of incident light can mean very little beyond the practical finding that ordinary diffuse laboratory light is effective in producing noticeable inhibition. This is true because the bacterial growth is limited to the surface, which is also the region of greatest absorption of light. Thus if sufficient light is absorbed at the surface layer to activate 90% of the dye molecules in this region, a great increase in intensity could not greatly increase the inhibitory effect on bacterial growth, although it might produce more change at greater depth. We have found that the inhibitory effect can be removed by washing the surface of the agar, indicating that the effects are generally limited to this region. This may also be a function of the O_2 concentration, which must be greatest at the surface, since O_2 is a necessary component of the common photochemical reaction of these dyes.[1, 2]

With more intense irradiation, *e. g.*, exposure to direct sunlight, the plates are definitely bleached. The destructive effects of previously irradiated fluorescein dyes, to which group both eosine and erythrosine belong, has been demonstrated by Moore[3] for the killing of sea urchin eggs, and by Blum[4] for the hemolysis of erythrocytes. Blum and Spealman[5] have presented evidence to show that the destructive action is associated with the bleaching of the dye but not with the presence of H_2O_2, although this latter substance is formed.[2]

In all these experiments the light passed through the pyrex or glass covers of the Petri dishes, which remove the ultra-violet wave lengths ordinarily active on organisms and media. Thus the effects are undoubtedly due to the activation of the dye molecules, principally by visible light, and the phenomenon must be classed with photodynamic action.[6]

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1 Blum, H. F., and Spealman, C. R., *J. Phys. Chem.*, 1933, **37**, 1123.

2 Blum, H. F., and Spealman, C. R., *Am. J. Physiol.*, 1934, **109**, 605.

3 Moore, A. R., *Arch. di Sci. Biol.*, 1928, **12**, 231.

4 Blum, H. F., *Biol. Bull.*, 1930, **58**, 224.

5 Blum, H. F., and Spealman, C. R., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 1007.

6 Blum, H. F., *Physiol. Rev.*, 1932, **12**, 23.

Further work is in progress and the present preliminary note is published merely to call attention to the possible error that may be introduced by failure to guard bacterial media containing photo-active dyes from light.

7785 P

Effect of Thyroxin Injections upon the Feather of the Guinea Fowl.

MARY HARDESTY. (Introduced by H. N. Gould.)

From the Department of Biology, Newcomb College, Tulane University, and Whitman Laboratory of Experimental Zoology, The University of Chicago.

This paper presents a preliminary report of an analysis of the effect of hypernormal concentrations of thyroxin upon an embryonic organ, the feather germ.

The writer has demonstrated previously[1] that the feather pattern of the guinea is formed by repetition of a pattern unit, a transverse row of spots, which recurs after the lapse of a definite period of time, constant for an individual bird. This pattern period may be calculated, and furthermore the beginning and the end of a period may be distinguished easily in a definitive feather or in a growing germ.

In the experiments to be reported, 10 guinea fowls, 5 males and 5 females, were injected with solutions of Squibb's crystalline thyroxin in single injections as well as double and triple injections separated by 4 to 9 days, the doses ranging from 2 to 6 mg. The birds weighed between 2½ and 3 lbs. Juhn and Barnes[2] produced an area of abnormal black pigmentation in brown Leghorns with doses as small as 0.5 mg. Although the guinea is a smaller bird, doses of thyroxin 12 times as large as this failed to produce any analogous change in pigmentation. On the other hand, doses less than 12 mg. cause no molting in the brown Leghorn,[3] while in the guinea 4 mg. is sufficient to produce general molting. No sex differences were noted in the susceptibility of guinea feathers to thyroxin.

Comparison of large areas of feathers treated with 4 mg. of thyroxin with their predecessors and successors grown from the

[1] Hardesty, Mary, *J. Exp. Zool.*, 1933, **66**, 53.

[2] Juhn, Mary, and Barnes, B. O., *Am. J. Physiol.*, 1931, **98**, 69.

[3] Lillie, F. R., and Juhn, Mary, *Physiol. Zool.*, 1932, **5**, 124.

same follicles—subcutaneous injections of India ink identified individual follicles—showed that the thyroxin feathers broke more easily, that they were shorter, that they carried fewer barbs, and that they required a shorter time to reach maturity. The contour of the feathers was concave, due to shorter barbs in the affected region.

Injections of thyroxin left the pigmentation period unaltered. However, minor but definite transpositions occurred in the pattern of a feather growing during the presence of hypernormal concentrations of thyroxin in the blood stream. The pattern modifications extended over 2 pattern periods.

Feathers whose tips were grown under the influence of thyroxin were more blunt in outline than controls, and the initial spot was shorter along the long axis of the feather, and at the same time broader laterally. When the thyroxin effect reached the feather at a lower level on the vane the spots of the first affected pattern period were smaller and lay closer to the transverse row above than in the controls; in the second pattern period the spots were larger and lay farther from the row above.

Measurements under the compound microscope with the aid of a mechanical stage showed that the barbs in the region affected by thyroxin were spaced first closer together and then farther apart along the rachis than the corresponding barbs in controls. This difference in the spacing of the barbs, together with an increase in the growth rate of individual barbs, which was ascertained by the methods described previously, accounts for the changes in the shape and arrangement of the spots appearing in the pattern of feathers grown under the influence of thyroxin.

Various data, some of which have not been mentioned here indicate that the differences in barb spacing are in turn due to a more rapid deposition of barbs at the ventral side of the collar, and to 2 complementary changes in the growth rate of the rachis.

7786 P

Quantitative Determination of Free Cholesterol and Cholesterol as Esters Without Digitonin.

JOHN G. REINHOLD. (Introduced by A. J. Quick.)

From the Biochemical Laboratory, Philadelphia General Hospital.

Cholesterol esters react more rapidly than free cholesterol with sulfuric acid and acetic anhydride to form the characteristic green color of the Liebermann-Burchard reaction. This difference, first observed for cholesterol palmitate[1] has been found to hold for the acetate and oleate ester as well. This difference in the reactivity of the free and combined cholesterol influences the results obtained by the usual colorimetric methods, and it is certain that in several of the widely used procedures true color equivalence is not attained.

By developing the Liebermann-Burchard reaction at 0-2°C., only the esters will develop color while the free cholesterol remains practically colorless, thus allowing the determination of cholesterol esters in the presence of free cholesterol. Then by completing the reaction at 38°C., total cholesterol can be determined in the same solution. This procedure, which obviates the use of digitonin, is carried out as follows:

Alcohol-ether extracts prepared according to Bloor,[2] containing the equivalent of 1 cc. of blood serum, are evaporated to dryness at a temperature of 60-70°C. The dry residue is extracted with anhydrous chloroform* which is filtered through cotton into a glass-stoppered 10 cc. cylinder. Two standard solutions, one containing cholesteryl oleate equivalent to 1.6 mg. cholesterol† in 10 cc. chloroform, the other cholesterol in the same amount and volume, are measured into similar cylinders. Both standard and unknown solutions are cooled in a refrigerator for 10 minutes. Acetic anhydride and sulfuric acid are mixed in the proportion of 1 cc. of acetic anhydride to 0.025 cc. of concentrated sulfuric acid, then cooled to 0-2°C. One cc. of the cold mixture is added to the cooled standard and unknown solutions. After mixing these are placed in an ice bath

[1] Reinhold, J. G., *J. Biol. Chem.*, 1934, **105**, lxxi.

[2] Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry, Vol. 2, Williams and Wilkins, Baltimore, 1932.

* Commercial chloroform should be washed, dried over anhydrous potassium carbonate and distilled. The distillate is dehydrated with phosphorus pentoxide and again distilled. The same chloroform should be used in standard and unknown solutions.

† 2.7 mg. cholesteryl oleate contain 1.6 mg. cholesterol.

at a temperature of 0-2°C. After 40-50 minutes, the unknown solutions and both standard solutions are compared in a colorimeter with an artificial standard consisting of an aqueous 0.0025% solution of naphthyl green B. A red eye-piece filter like that used by Bloor is employed in making the readings. Color that appears under the conditions described originates with esterified cholesterol, although a little color is developed by free cholesterol.

The solutions next are warmed in a water bath at a temperature of approximately 38°C. for about 40 minutes, and then are allowed to stand at room temperature for 10 minutes. A slight precipitate that often appears in the unknown solutions settles out or can be removed by centrifugation. The clear solutions are compared colorimetrically with either the cholesterol or cholesteryl oleate standards. On warming, free cholesterol rapidly develops color so that esterified and free cholesterol yield equivalent concentrations of color at this time. From readings made after the solutions have been warmed, the total cholesterol may be calculated by the usual formula.

Ester cholesterol is calculated from the first readings. Since free cholesterol, if present, adds slightly to the color developed by esterified cholesterol in this stage of the reaction, a correction is applied. It is calculated on the assumption that cholesterol of either type develops a basal intensity of color equivalent to the amount formed by free cholesterol, while esterified cholesterol produces an additional quantity. The latter is proportional to the concentration of ester, so that

$$\text{Ester cholesterol in milligrams} = \frac{B-C_2}{O-C_1} \cdot S$$

where B = reading of naphthyl green solution against the unknown,

O = reading of naphthyl green solution against cholesteryl oleate standard,

S = concentration of cholesterol in cholesteryl oleate standard,

C_1 = reading of naphthyl green against standard cholesterol solution,

$$C_2 = \frac{\text{total cholesterol in unknown solution}}{\text{cholesterol in standard solution}} \cdot C_1.$$

If more than 2 unknown solutions are to be read, it is advisable to prepare 2 sets of standards, one pair being read before and the other after reading the unknown solutions against the artificial standard. The time of each reading is recorded and the readings of the cholesteryl oleate and free cholesterol standards corresponding to the time at which each unknown was read can be obtained by interpolation.

Coprostenol (allocholesterol), which reacts under these conditions like esterified cholesterol rather than free cholesterol, apparently is not present in unbound form or exists in serum in concentrations too low to interfere with the application of the differential reaction.

Known mixtures of cholesteryl oleate and cholesterol have been analyzed correctly by the procedure described. Cholesteryl oleate added to alcohol-ether extracts of serum can be recovered with reasonable accuracy. Several comparisons of determinations of cholesterol and cholesterol esters by the new method and by a gravimetric digitonin procedure[3] indicate that the results agree closely.

7787 C

Diuresis of Hyperthyroidism.

A. S. DIX, J. M. ROGOFF* AND B. O. BARNES.

From the Department of Physiology, The University of Chicago.

During the course of investigations on diabetic animals it was observed that thyroid administration or injection of anterior pituitary extracts did not produce diuresis in pancreatectomized dogs. In a previous report[1] it was shown that an extract from the anterior pituitary, which caused marked diuresis failed to produce such an effect in thyroidectomized animals. The results indicated that the induced hyperthyroidism was responsible for the polyuria. This work was confirmed by Biasotti.[2] It was also observed[3] that if the pituitary and pancreas were previously removed, thyroid administration failed to cause the usual diuresis.

Hyperthyroidism was induced by feeding desiccated thyroid (Armour's), in doses of 1 gm. per kilo body weight per day or by injecting the anterior lobe extract previously described[1] for 7-10 days. In some cases both methods were used in the same animal, considerable time elapsing between each experiment. Twelve dogs were used, in which the following operations were performed:

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[3] Ewert, B., *Biochem. Z.*, 1933, **263**, 149.

* Aided by a grant from the Commodore Beaumont Foundation.

[1] Barnes, B. O., Regan, J. F., and Bueno, J. G., *Am. J. Physiol.*, 1933, **105**, 559.

[2] Biasotti, A., *Rev. Soc. Argentina Biol.*, 1933, **9**, 499.

[3] Barnes, B. O., *Am. J. Physiol.*, 1934, **109**, 5.

(a) 1, hypophysectomy.
(b) 3, hypophysectomy and pancreatectomy.
(c) 3, pancreatectomy.
(d) 1, bilateral suppression of epinephrine secretion.
(e) 1, pancreatectomy and unilateral suppression of epinephrine secretion.
(f) 3, pancreatectomy and bilateral suppression of epinephrine secretion.

The 24-hour urine specimens were collected in ordinary metabolism cages.

TABLE I. Showing the Daily Urine Output (cc.) of
A. Hypophysectomized dog; thyroid feeding.
B. Normal dog; injection of anterior lobe extract.
C. Pancreatectomized dog with bilateral suppression of epinephrine secretion; thyroid feeding.
The first 3 observations on dogs A and C are controls.

A	B	C
500	—	580
320	—	570
630	—	340
400	300	290
530	200	560
700	780	450
1330	1010	470
1360	1020	210
700	1080	740
1400	1340	470
1180	1350	740
1170	1120	600

Some typical results are illustrated in Table I. In hypophysectomized dogs (A), as in normal animals, thyroid administration causes diuresis. This polyuria is similar to that observed after the injection of anterior lobe extracts into normal dogs (B), as previously reported. The remaining animal (C) illustrated in Table I was operated as described in group (f). It is obvious that the urine output was not increased during the period of thyroid administration. More than 5 months prior to the thyroid administration experiment, this animal was given a series of daily injections of anterior pituitary extract. Diuresis did not result from these injections. In all of the 10 pancreatectomized dogs, diuresis failed to occur following administration of either desiccated thyroid or anterior lobe extract. In another animal (d), subjected to bilateral suppression of epinephrine secretion but not to pancreatectomy, thyroid administration was followed by polyuria.

Since some polyuria develops after pancreatectomy, the question might arise whether this masks any increase due to thyroid

feeding. We feel that this is not the explanation of our negative results. In some of our pancreatectomized dogs the urine output was not more than in normal dogs of similar weight. Further, the polyuria observed after thyroid feeding is much greater than that in any of the diabetic dogs in the above series.

In some of the pancreatectomized animals the B.M.R. and heart rate were observed during thyroid administration or anterior pituitary injections. In each case a typical rise occurred, which suggests that the diuresis is not the result of accelerated metabolism. Further work will be necessary to explain the polyuria of hyperthyroidism and its absence in pancreatectomized animals.

Summary. The diuresis produced in normal animals by thyroid administration fails to occur in pancreatectomized dogs although the metabolic rate is increased.

7788 C

Van den Bergh Reaction of Bilirubin in Xanthochromic Cerebrospinal Fluid.

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From the Buffalo General Hospital and University of Buffalo Medical School.

It is well recognized that in blood serum normal bilirubin within certain limits of concentration gives a delayed reaction in the Van den Bergh test (diazo reaction). This type of reaction differs from the prompt reaction obtained with isolated bilirubin and that encountered in a variety of pathological states. In attempting to explain this difference numerous factors have been considered. The most thoroughly studied of these concern changes in chemical constitution of the bilirubin molecule, changes in adsorption of the bilirubin by blood colloids and bilirubin concentration. None of the explanations has been universally accepted.

An important reason for the lack of conclusive experiments which would solve this problem is that much of the work has been done on blood where it is practically impossible to control all of the factors properly. Not only are the colloids in relatively high concentration, but also the influence of the liver can not be excluded entirely even in dehepatized animals.

It would seem that much information could be gained from a study of the phenomena under less complicated conditions. Such conditions are found in the cerebrospinal fluid. Influence of the

liver can be excluded almost entirely and the colloids (proteins) are present normally in such low concentration as to be practically negligible. Even the concentration present under extreme pathological conditions is very low when compared with that normally present in the blood.

Soon after a small quantity of blood is introduced into the ventricles of the brain or into the subarachnoid space the cerebrospinal fluid becomes xanthochromic. Obviously the color is produced by a pigment derived from hemoglobin and is undoubtedly identical with bilirubin formed elsewhere in the body including that normally present in the blood serum.

The present paper deals with the type of Van den Bergh reaction obtained in 20 samples of xanthochromic cerebrospinal fluid taken from 15 patients. In the majority of the patients the cause of the xanthochromia was subarachnoid hemorrhage. In no case was the patient jaundiced. That no appreciable amount of bilirubin was introduced directly with the blood was determined in several of the cases where preliminary samples were taken soon after the hemorrhage and before xanthochromia developed.

The technique of the Van den Bergh was the same as that employed with blood serum except that a control tube of the test fluid diluted with distilled water was used as a blank to offer a contrast with the color reaction. This was necessary because the amount of pigment present was very small. The time of first appearance of pink color and that of its maximum development were noted. Quantitative determinations were made also whenever the degree of color was great enough to match with the standard. Protein content was measured roughly by the use of the Pandy and nitric acid ring tests.

In 13 of the 20 samples (65%), the color appeared promptly after the addition of the reagent. The full color developed in from 1 minute to 2 hours. These reactions corresponded with the "prompt direct", "prompt biphasic" and the "delayed biphasic" reactions of the literature but for the sake of simplicity they will be grouped together as "prompt" reactions in this discussion. The remaining 7 samples gave reactions that were delayed from 2 to 30 minutes. They attained their maximum colors within 2 hours.

By comparing the type of reaction with the concentration of pigment it was found that in every case where the concentration exceeded 0.3 mg. % a prompt reaction occurred. When the concentration was lower the types of reactions were equally divided between prompt and delayed.

In a similar manner the type of reaction was compared with the protein concentration. In all cases where the protein concentration was within normal limits the reaction was prompt. When the concentration was above the normal the type of reaction varied.

However, when the results were tabulated in such a way that there was a progressive increase in the pigment concentration with increasing protein concentrations for each pigment value the correlation was quite definite. Prompt reactions occurred in all cases where the protein concentration was normal and also where the pigment concentration was high regardless of the protein content. When the pigment level was relatively low and the protein content high the reactions were predominantly delayed.

From these results it can be concluded that the type of Van den Bergh reaction of the bilirubin in xanthochromic cerebrospinal fluid depends upon a reciprocal relationship between the pigment and protein concentrations. While it cannot be said definitely that the same relationship holds true in blood serum there are good reasons for believing that such may be the case. It seems significant that reactions quite similar to those described are found in the blood of many cases of anemia associated with over production or retention of bilirubin. It is quite possible that these reactions are comparable directly with those in this series where the pigment concentration was increased disproportionally over the protein concentration. This would lend support to the view set forth by Barron,[1] that the bilirubin of normal blood serum is prevented from reacting promptly by adsorption on colloids (proteins) and that its ability to react in this manner is restored when there is failure of complete adsorption. In the opinion of the authors this renders it unnecessary to introduce other factors such as the influence of liver cells to explain the prompt reactions in retention jaundice.

[1] Barron, E. S., *Medicine*, 1931, **10**, 77.

7789

Further Studies upon Development of Somatic Activity in Albino Rat Fetuses.

A. W. ANGULO Y GONZALEZ. (Introduced by H. H. Donaldson.)

From the Wistar Institute of Anatomy and Biology, Philadelphia.

As previously reported[1] the early development of response to stimulation in albino rat fetuses passes through 3 stages: myogenic, neuromotor, and sensory motor. Experiments are here reported which were designed to test in a more definite manner the first phase of the development of the response to stimulation, the myogenic stage.

The myogenic response was described[1] as follows: "The movements elicited by strong mechanical stimulation are slow and feeble, but maintained. The responses elicited by light tactile stimulation, reflex activity, are also slow and feeble, but quickly followed by relaxation." One may justly infer that maintenance of contractions is indicative of purely myogenic activity. This is not the case. The above statement is true only in relation to the strength of the stimuli used, not to the stage of development. In further experiments in which 80 fetuses from 12 different litters were studied, the mechanical stimulation was applied to the fetus with different degrees of strength. The result shows that when the stimulus was just strong enough to elicit responses, these responses were not maintained, but instead were quickly followed by relaxation. If the strength of the stimulus was increased, the contractions began to be maintained and the length of maintenance increased with the increased strength of the stimulus. However, a limit was reached beyond which this period of maintenance ceased to increase, even when the stimulus applied was strong enough to seriously damage the tissue.

Since with adequate though light stimuli the contractions were followed by quick relaxation, there seems to be, upon this basis, no marked difference between the nature of myogenic and reflex activity. Thus, at this early stage of response to stimulation the following properties occur: Latent period, summation, and contractions followed by quick relaxation are common to both myogenic as well as reflex activity. One might conclude that the earliest reaction, previously described as myogenic, might be, in fact, reflex. In order to test this the following experiments have been performed with 48 fetuses taken from 9 different litters: The fetuses were tested first

[1] Angulo y Gonzalez, A. W., Proc. Soc. Exp. Biol. and Med., 1933, **31**, 111.

for reflex activity by stimulating them lightly with a hair upon the snout and other areas. The younger fetuses (360 to 370 hours) did not respond to this type of stimulation. The fetuses of 378 hours to about 380 hours responded to stimulation of the snout only, and some older than 390 hours gave responses to stimulation of the snout and also the fore limb. After the fetuses had been tested for tactile responses and the results recorded, they were lightly stimulated with a porcupine quill about the region of the fore limb. The fetuses of about 360 hours did not respond. However, all the older fetuses responded, and, the responses varied in quality in respect of the strength of the stimulus. They were quick reflex-like movements when the strength of the stimulus was just adequate; and maintained responses when a stimulus somewhat stronger was applied. After these observations had been made and recorded, the fetuses were curarized.

One-tenth cc. of a 1% solution of curare injected intraperitoneally in new born rats was sufficient to produce complete curarization in 10 minutes, as indicated by complete disappearance of reflexes. This procedure was used as a norm for my early experiments. However, in the younger fetuses this dose proved to be too strong, and several of the fetuses died of heart failure, also this quantity of fluid caused great distension. Further tests were made using fetuses 17 days after insemination, to determine a more adequate norm. It was found that 0.05 cc. of a 1% solution was sufficient to cause complete curarization in 3 minutes. This new norm has been consistently used with satisfactory results.

Three minutes after the injection, the fetuses were again tested for reflexes, but the results were negative. They were then lightly tapped with the quill about the region of the fore limb, and responded in a manner identical with that observed before curarization. That is, when lightly tapped, the movements were reflex-like, and the contractions were maintained when the stimulus was stronger.

Since curarization completely destroys the physiological continuity between the central nervous system and muscles, these experiments confirm my earlier conclusion, namely, that the early response to stimulation in the albino rat fetuses induced by direct stimulation of the muscles is purely myogenic in nature.

7790 P

The Kolmer-Wassermann Test with Monkey Serum.

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From the Research Institute of Cutaneous Medicine, Philadelphia.

In the conduct of complement fixation tests for poliomyelitis antibody[1] we have conducted Wassermann tests with the sera of 25 monkeys and found that all gave completely negative reactions.

It would appear, therefore, that monkey sera do not yield the non-specific positive Wassermann and other complement fixation tests sometimes yielded by normal rabbit, dog and mule sera.[2]

The tests were conducted according to the technic of the Kolmer modification of the Wassermann reaction[2] employing a cholesterolized and lecithinized alcoholic extract of beef heart as antigen in dose of 10 antigenic units.

Each serum was heated in a water bath at 55°C. for 30 minutes and used in amounts of 0.1, 0.05 and 0.025 cc. with 0.1 cc. in the serum controls. An antisheep hemolytic system was employed with a primary incubation of 15 hours at 4°C. followed by 10 minutes in a water bath at 37°C.

The 25 sera included 2 from normal monkeys, 5 from monkeys with the paralysis of acute anterior poliomyelitis, 7 from monkeys immunized about 14 months previously by 10 subcutaneous injections of the Kolmer vaccine of attenuated monkey spinal cord virus and 11 from monkeys inoculated intracerebrally with neutralized mixtures of serum and virus.

On the basis of the results it may be stated that the sera of normal and poliomyelitic immune monkeys as well as those with poliomyelitis do not give non-specific positive Kolmer-Wassermann reactions.

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[1] Kolmer, J. A., and Rule, A. M., *J. Immunol.*, in press.

[2] Kolmer, J. A., Serum Diagnosis by Complement Fixation, Lea and Febiger, 1928.

7791 C

Scalp Products and Hair of Men and Women as Culture Media for Certain Pathogenic Fungi.*

JOHN W. WILLIAMS.

From the Homberg Memorial Infirmary and the Department of Biology and Public Health, Massachusetts Institute of Technology.

A study was made of the growth of pathogenic fungi on hair and scalp products before puberty, as collected and after extraction with ether in a Soxhlet for 24 hours.[1] A similar study of hair and scalp products of men and women is here reported. Care was taken that this hair was clean, untreated and undyed. The hair of numerous individuals of different ages was mixed. About 6 cc. of hair was placed in test tubes, covered with distilled water, the tubes plugged with cotton and autoclaved.

The following pathogenic fungi and 2 non-pathogenic saprophytes *Lichthemia sp.* and *Scopulariopsis brevicaulis* were studied: *Achorion schoenleinii, Acladium castellani, Candida candida, Endodermophyton tropicale, Endomyces capsulatus, Endomyces dermatitidus, Epidermophyton cruris, Epidermophyton inguinale, Glenospora gammeli, Geotrichum bachmann, Indiella americana, Microsporon audouini, Microsporon felincum, Microsporon gypseum, Monosporum apiospermum, Monilia albicans, Oöspora humi, Sporotrichum schenkii, Trichophyton crateriforme, Trichophyton granulosum, Trichophyton gypseum asteroides, Trichophyton gypseum lacticolor, Trichophyton interdigitale, Trichophyton japonicum, Trichophyton niveum, Trichophyton sulfureum, Willia anomala.*

In reporting results W is used to designate women's hair, M men's, EW extracted women's hair, EM extracted men's. All organisms studied showed growth on our stock 4% peptone, 1% dextrose, 1½% agar medium in 3 days. Growth was at room temperature in diffuse light.

Acladium castellani, Candida candida, Geotrichum bachmann, Lichtheimia sp., Microsporon felineum, Microsporon gypseum, Monilia albicans, Oöspora humi, Sporotrichum schenkii, Trichophyton granulosum, Trichophyton gypseum asteroides, Trichophyton gypseum lacticolor, Trichophyton interdigitale, Trichophyton japonicum, Trichophyton niveum, Willia anomala showed growth on all hair media in 3 days. *Endodermophyton tropicale, Epidermophyton*

* Contribution No. 45 from the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Mass.

[1] Williams, John W., Proc. Soc. Exp. Biol. and Med., 1934, **31**, 944.

cruris, Epidermophyton inguinale, Glenospora gammeli, Microsporon audouini, Monosporum apiospermum, Trichophyton crateriforme, Trichophyton sulfureum showed growth within 8 days. On W and M *Endomyces capsulatus, Endomyces dermatitidus* and *Indiella americana* showed good growth in 8 days and on EW and EM scant growth in 18 days. On W and M *Scopulariopsis brevicaulis* showed good growth in 3 days and on EW and EM good growth in 10 days (saprophytes of the groups Actinomyces, Alternaria. Aspergillus, Fusarium, Homodendron, Mucor and Penicillium were checked and showed good growth on all media in 3 days). *Achorion schoenleinii* showed good growth on EW and EM in 3 days and scant growth on W and M in 10 days. No growth of the latter organism had been noted on children's hair.[1]

No inhibition of growth of *Microsporon audouini* was noted on any of the media as one might expect because of lack of pathogenicity of this organism for scalps of adults. Future work will be directed toward isolation of fungi from healthy scalps under the assumption that infection may be spread by scratching or allergy result from absorption or inhalation of the organismal specific substances.

7792 C

III. Effect of Dyes on Colonies of Certain Pathogenic Fungi.*

JOHN W. WILLIAMS AND LEO GREEN.

From the Department of Biology and Public Health and the Homberg Memorial Infirmary, Massachusetts Institute of Technology.

Observations were made on growth and coloring of colonies of certain pathogenic fungi cultured on a medium (4% peptone, 1% dextrose, 1½% agar, pH 5.6) containing alcoholic nigrosine, litmus, eosin Y and eosin B, respectively.[1, 2]

In the work here reported 5 batches of a similar medium containing the following percentages of dyes† respectively, 2% fluores-

* Contribution No. 46 from the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Mass.

1 Williams, John W., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 1173.

2 Williams, John W., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 1174.

† Fluorescein, Schultz No. 585, Lot No. 6054. Eosin Y, Schultz No. 585, Batch E-7. Methyl Blue, Lot No. 3421. Janus Green, C. I. No. 133. Neutral Red, Lot No. 7272. Wright's Stain, NWr-7. All manufactured by National Aniline and Chemical Company, New York.

TABLE I.

Organism	F	M B & E	Wr.	N R	J G
Achorion schoenleinii	3 ++++ y	3 +++ r	8 ++	45 t. r.	—
Acladium castellani	3 +++— g	5 +++ b.p.	5 ++ cb	5 ++ p	8 ++ yr
Candida candida	3 +++ g	3 ++++ p—b	3 +++ s—b	3 ++ y—p	45
Endodermophyton tropicale	7 +++ g	18 ++ b	45 ++	10 ++ p	45 + b
Endomyces capsulatus	3 +++ g	10 +++ p		18 +	—
Endomyces dermatitidus	3 ++++ g	10 ++++ p	45 +	3 + r	45 ++ p
Epidermophyton cruris	3 +++ g	3 +++ p	10 ++	3 +	—
Epidermophyton inguinale	3 ++++ y	5 ++++ p—	3 +	3 +	—
Glenospora gammeli	3 +++ yg	3 +++ p	3 +++	45 ++	
Geotrichum bachmann	3 ++++ g	3 +++ b	3 ++++ b.s.	3 ++++ r	
Indiella americana	3 +++ g	5 +++	45	45 ++ r	
Lichthiemia sp.	3 ++++ yt	3 ++++	3 ++++	3 ++++	6 ++++
Microsporon audouini	8 +++ y	8 +++ p		8 +	
Microsporon felineum	3 +++ yt	3 +++ pt	5 ++	3 ++	8 ++ t
Microsporon gypseum	3 ++++ s—y	3 ++++ p	7 ++ cb—y	3 ++ p	45 + b
Monilia albicans	3 +++ g	3 +++ b	3 +++ cb—s	6 +++ r	
Monosporum apiospermum	7 ++++ g	3 +++ p	10 ++++	8 ++ r	45 ++ t
Oöspora humi	3 ++++ g	3 ++++ pv	3 ++++ b	3 ++++ r	
Scopulariopsis brevicaulis	3 ++++ gs	3 +++ vp	6 +++ c	8 +++ p	10 ++ s
Sporotrichum schenckii	3 +++ g	13 +++ b	3 ++ c—p—b	5 ++ r	
Trichophyton crateriforme	3 +++ y	3 +++ r		5 + p	
Trichophyton granulosum	3 ++++ y	3 ++++ p	3 +	7 +	
Trichophyton gypseum lacticolor	3 ++++ y—	3 ++++ r—	7 ++	7 ++ pt	
Trichophyton interdigitale	3 ++++ y—	3 ++++ p—	3 +++	3 ++	
Trichophyton niveum	3 ++++ yt	3 ++++ r	3 +	3 ++	
Trichophyton sulfureum	7 +++ y	3 +++ —p—	18 ++ t	10 ++ pt	
Willia anomala	3 +++ y	10 ++ b		6 ++ r	6 ++ r

cein, 1% methyl blue and 1% eosin Y, ½% neutral red, ½% janus green, ½% Wright's stain suspension, were studied. The organisms were grown in diffused light at room temperature and observed over a period of 45 days.

The following pathogenic fungi and 2 non-pathogenic saprophytes, *Lichthiemia sp.* and *Scopulariopsis brevicaulis,* were observed: *Achorion schoenleinii, Acladium castellani, Candida candida, Endodermophyton tropicale, Endomyces capsulatus, Endomyces dermatitidus, Epidermophyton cruris, Epidermophyton inguinale, Glenospora gammeli, Geotricheum bachmann, Indiella americana, Microsporon audouini, Microsporon felineum, Microsporon gypseum, Monilia albicans, Monosporum apiospermum, Oöspora humi, Sporotrichum schenkii, Trichophyton crateriforme, Trichophyton granulosum, Trichophyton gypseum lacticolor, Trichophyton interdigitale, Trichophyton niveum, Trichophyton sulfureum, Willia anomala.*

Table I gives the time of appearance of growth and its relative amount. The following abbreviations are used: F, fluorescein; MB & E, methyl blue and eosin Y; Wr., Wright's stain suspension; NR neutral red; JG janus green; b, blue; br, brown; c, copper colored; g, golden; p, pink; r, red; s, salmon; sc, scarlet; t, tinted; v, varies; y, yellow; y— (letter followed by dash) indicates color centrally; —y— (dash letter dash) indicates color between center and border.

Reference to the chart will reveal that in the majority of cases growth is more profuse and more frequently colored when the acid dyes, methyl blue, eosin Y and fluorescein are used. With few exceptions, in keeping with the greater toxicity of basic dyes, growth occurs either not at all or to a lesser extent on media containing them. The strongly basic dye, janus green, shows this effect to the greatest degree, whilst Wright's stain suspension containing a low concentration of basic dye is less active and the weakly basic dye, neutral red, least.

The letters are used to designate color. Growths showing color on neutral red do not show the blue indicative of alkaline reaction. On Wright's 2 colors are noted in several colonies. On methyl blue and eosin Y, some growths show blue, some pink, some both. Selectivity of the latter dyes might be variable since both are strong acid dyes.

In practically all instances on acid dye media, growth and differentiation were as good as on the control. If acid dye media were selected for routine work much smaller concentrations of dye could be used. Such media are especially valuable for contrast.

Colonies which show color macroscopically also show it microscopically. The elements (*i. e.*, portions of the cells) of growth colored are similar to those colored in staining growths from ordinary media. Double staining was noted in a few instances in growths from methyl blue-eosin Y and Wright's stain suspension media. The microscopic picture of dye media growth was in several instances superior to that obtained when specimens were stained from growth on ordinary media. If dye could be prevented from diffusing out of dye media cultures without destroying structure it is probable that the microscopic picture would be much more desirable.

7793 C

Experimental Production of Bulbar Poliomyelitis.

JOHN A. TOOMEY.

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Poliomyelitis has been experimentally produced in *Macacus rhesus* monkeys by injecting the virus into the ileum between clamps or subserosally in the same area.[1] Levaditi, Kling and Hornus[2] introduced the virus into the gastrointestinal tract of a *Macacus cynomolgus* monkey and the animal later became ill with the disease. In the former instance,[1] the clinical picture was similar to that seen in the human being ill with spinal poliomyelitis. The animals developed palsy of muscle groups, monoplegia and paraplegia of the legs, but not the condition of quadriplegia that usually follows intrasciatic and intracerebral inoculations and intranasal instillations.

It has been shown that the virus spreads along the axis cylinders of the sympathetic thoracolumbar outflow.[3] It has also been pointed out that such a spread from the gastrointestinal tract directly to the cord by way of the afferent and efferent grey fibers has a simple and logical anatomical explanation.[4] It is an explanation that would also apply to typhoid fever, another gastrointestinal disease.[5] Al-

[1] Toomey, John A., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 680.

[2] Levaditi, C., Kling, C., and Hornus, G., *Compt. rend. Soc. de biol.*, 1933, **112**, 43; *A. J. Dis. Child.*, 1934, **48**, 423.

[3] Toomey, John A., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 502, 702.

[4] Toomey, John A., *Jaahrb. für Kinderheilkinde*, in press; *Am. Coll. Physicians*, April 18, 1934; *Annals Int. Med.*, in press.

[5] Toomey, John A., *A. J. Dis. Child.*, 1934, **48**, 1296.

though the hypothesis as to the mechanism of spread of the virus holds true in the average case of the spinal type of poliomyelitis, it cannot be used to explain the mechanism of the production of bulbar paralysis.

Histopathological examination of the autopsied material revealed findings similar to those of Harbitz and Scheel[6] in that I found marked involvement of the nuclei of the brain stem and medulla, especially in grey fibered areas, as well as increased vascular markings. One was not always fortunate enough to see cases of so-called bulbar poliomyelitis from the onset, but the histories, symptomatology and objective findings of 45 of the 440 cases admitted to Cleveland City Hospital all presented such evidence that it could be stated that the initial involvement in practically every instance was vagal in character, *i. e.,* accompanied by vomiting, followed by dysphagia, dysarthria and aphonia with other symptoms developing later to indicate a spread to other cranial nerves. The fact that the vagus is the motor nerve to the small intestine made it logical to suppose that absorption of the virus could take place not only along the thoracolumbar outflow, but, in some cases, along the vagus nerve as well, and that the spread of the virus in cases manifesting bulbar symptoms was along the vagal nerves directly to the nucleus ambiguous on both sides and from there to other locations.

The object of these experiments was to produce the disease in *Macacus rhesus* monkeys by way of the vagus nerve, to note the symptoms and signs in these animals and to determine if the histopathological picture was like that found in the human being who died of bulbar involvement.

Several postmortem examinations showed that the right vagus nerve is slightly larger than the left and perhaps easier to approach from an operative standpoint. Operations were performed on 2 animals under general anesthesia. The vagus was exposed just lateral to the isthmus of the thyroid, the nerve separated from the carotid sheath and irritated by clamping it a few times with a Kelly hemostat. Electrocardiograph records showed only a tachycardia.

Eight animals were used for the next experiment. The right vagus nerve was exposed and irritated by clamping it a few times with a hemostat; 0.2 cc. of a 1% solution of virus was injected. The needle, 30 gauge, (made specially by Vim McGregor Instrument

[6] Harbitz, F., und Scheel, O., *Pathologisch-anatomische Untersuchungen über akute Poliomyelitis und verwandte Krankheiten von den Epidemien in Norwegen,* 1903-1906. *Videnskabs-Selskabets Skrifter, I. Math-naturv. Klasse.* 1907. No. 5. Christiania. In Kommission bei Jacob Dybwad. 1907.

Co.) was drawn back and forth inside the sheath to expose and further irritate the axis cylinders. The nerve was ballooned out after the injections. The vagus is very small, but if its epineurium is not cracked the dose mentioned can be injected if it is done slowly over a period of from 3 to 5 minutes.

Monkey No. 263 looked sick on the 4th day; he had anorexia; he could jump up and down but could not hold up his head. When the head was moved, it flopped back to the chest wall and the right arm hung flaccid. There was furring also. On the 5th day, the animal's cry was hoarse. When food or fluid was placed in the posterior pharynx he could not swallow and he was extremely restless. He died suddenly on the 6th day without having shown any evidence of paralysis of the legs. Grossly, the postmortem examination was negative.

Monkey No. 268 was active for 3 days before he became sick; the right arm and legs became weak and there was furring. The entire right side became paralyzed. On the 8th day he was found dead. His cry was always shrill and clear. At autopsy a massive bilateral pulmonary tuberculosis complicated the picture.

Monkey No. 269 became sick, his cry was hoarse and there was furring on the 3rd day. He also had tachycardia and cyanosis of the mucosa of the lips and mouth. On the 6th day, he could neither cry nor swallow, his head hung limp on his chest and both arms were flaccidly paralyzed. He could jerk himself up to a standing position, however. He died that night. Grossly, postmortem examination showed marked dilatation of the vessels of the small and large intestines, a dilatation of the small intestine, an almost gangrenous area in one part of the large intestine, a central engorgement of the adrenals, and a solitary tubercle in one lung.

Monkey No. 271 was active the 1st and 2nd days after the operation. His cry was hoarse on the 3rd day. On the 4th day, he was weak; when he cried his voice was hoarse and aphonic, he could not swallow and there was furring. The right arm and leg were weak and he died that night. Grossly, the postmortem examination was negative save for marked vascular hyperemia of the small intestines.

Monkey No. 286 was active and apparently normal until the 9th day when he was found hunched up in a corner of his cage. There was furring and he was obviously ill. On the 10th day, he could not cry out or eat, and the muscles of the neck and both arms were paralyzed. When he stood up, his arms hung by his side. When his head was pushed about, it flopped back on his chest. When food

or fluid was put in the posterior pharynx he could not swallow. On the 11th day, both legs were paralyzed. He died this day. Grossly, there was nothing remarkable at autopsy save some pleuritic adhesions of the right lung.

Three other animals that were injected did not develop paralysis.

Objectively, monkeys Nos. 263, 269, 271, and 286 acted in a manner similar to that of human beings in that they evinced dysphagia, dysarthria and the paralysis of the muscles of the arm that is so commonly seen in cases of bulbar paralysis. This is easily understood, however, when it is remembered that the connection with the thoracolumbar outflow by way of the cranial sympathetic nerve is rather free and that an involvement of the phrenic nuclei is a possibility. Monkey No. 268 did not develop any bulbar symptoms apparently; even the electrocardiograph taken 10 minutes before death was recorded as being negative.

Postmortem sections were made through the lumbar, thoracic and cervical areas of the cord, through the inferior medulla, pontine angle, middle of the pons, superior and inferior colliculus, vermis, dentate nucleus, the basal nuclei and the motor cortex. They were stained by hematoxylin and eosin and Nissl's stain.

The histological results will be described more in detail elsewhere. Briefly, they were similar to those found in cases of bulbar poliomyelitis in human beings.

As the virus has an obligate affinity for grey fibers, it would be illogical to expect typical central vagal involvement in all experiments since the virus may just as well pass down as up the vagus nerve. The important point is that it can pass up the vagus nerve in some animals and experimentally produce the typical objective picture and the histopathological findings of bulbar poliomyelitis, thus lending further credence to the belief that the disease primarily originates from the gastrointestinal tract. If the virus runs up inside the vagus nerve until it loses its myelin sheath, one would expect to find the virus spilling over into the local grey matter of the medulla, pons and the base of the brain. Such an absorption along the vagi to the medullary area with secondary involvement of other nerve nuclei by contiguous extension explains the entire clinical picture in these cases of bulbar poliomyelitis in the same manner as absorption along the thoracolumbar outflow to the cord explains the usual spinal type of poliomyelitis.

7794 C

Protection of Monkeys Against Intracerebral Inoculation of Virulent Poliomyelitis Virus by Vaccination with Phenolized Poliomyelitis Vaccine.

D. MURRAY COWIE.

From the Department of Pediatrics and Infectious Diseases, University of Michigan Medical School and Hospital.

Because of the similarity of the pathological changes induced in the central nervous system by poliomyelitis and rabies viruses, and because of the well-known successful vaccination against rabies by means of the Pasteur method, I decided to carry out similar prolonged vaccination of monkeys against poliomyelitis virus, which was kindly furnished by Dr. Simon Flexner, and proved to be highly infectious for *Macacus rhesus* monkeys selected, through the courtesy of the Rockefeller Institute for Medical Research, as proper animals for such observations. The vaccine was prepared according to the Pasteur vaccine technique, by Dr. Herbert Emerson of the University Pasteur Institute, and consisted of a 5% suspension of emulsified cord tissue preserved in 0.7% phenol.

Monkeys were vaccinated subcutaneously by gradually increasing daily doses of vaccine over periods of 28 to 29 days, beginning with 0.10 cc. to 0.3 cc. and ending with 3.5 cc. to 4 cc., which doses in some instances were reached by the seventeenth day and continued at that point for the remainder of the vaccination period. A proved paralyzing dose of Flexner virus was then inoculated into the center of the frontal lobe of the brain either soon after (24 hours) or late after (30 days) the last dose of vaccine was given.

For example, one monkey (V. No. 2) inoculated 24 hours after the last dose of vaccine, developed transient evidences of the disease 23 days after intracerebral inoculation. His gait suddenly became awkward, he was unable to hold a carrot in his hands and easily lost his balance. Six hours later he was apparently normal and the following morning very active and remained so. Another monkey was given a larger dose of the same proved potent virus 30 days after the last dose of vaccine. No recognizable symptoms developed during the course of 3 months. A characteristic protocol is given in Table I.

Of 3 vaccinated monkeys 2 resisted intracerebral inoculation. The monkey that died was inoculated one month after the last dose of vaccine. The 2 vaccinated monkeys that resisted the first intracere-

TABLE I.
Vaccinated Monkey No. 2.

Date of Injection	Amount cc.	Location	Date of Injection	Amount cc.	Location
11-19-31	.10	Arm	12-10	2.25	Arm
11-20	.20	"	12-11	2.50	"
11-21	.30	"	12-12	2.50	"
11-23	.40	Thigh	12-13	2.50	"
11-24	.50	Arm	12-14	2.50	"
11-25	.60	"	12-15	2.75	" Irritable
11-26	.60	Leg	12-16	3.00	"
11-27	.70	"	12-17	3.00	"
11-28	.70	"	12-18	3.50	" Resists. Cries
11-30	.80	Arm	12-21	Has been looking ill since 12-18.	
12- 1	1.00	"	12-29	Has been normal last few days.	
12- 2	1.00	"	12-30	8:15 a. m. Ether anesthesia 1/3	
12- 3	1.50	"		cc. virus into frontal lobe. Wound	
12- 4	1.50	"		closed with sterile bone wax, su-	
12- 5	1.60	"		ture, gauze, collodion.	
12- 6	1.75	"	1 -22	Transient evidence of disease. Re-	
12- 7	1.75	"		covered.	
12- 8	2.00	"			
12- 9	2.00	"			

bral inoculation of virulent virus were inoculated intracerebrally a second time. One (V. No. 2), one month after the first inoculation, was given 0.5 cc. of a very heavy suspension of brain and cord tissue of a control monkey (a proved paralyzing dose) into the opposite lobe. On the third day there was a possibility of weakness of one hand; on the sixth day anorexia, inactivity, stooping forward, headache, watery eyes, slow movements; on the seventh day, progressive paralysis; *i. e.,* left lower extremity, right lower, left upper, complete helplessness, death on the sixteenth day (3-16-32).

The other monkey similarly protected against the first intracerebral inoculation 49 days later was started on a course of subcutaneous injections of living poliomyelitis virus. A 2% saline suspension was used. The injections varied from 0.02 cc. to 0.80 cc. given at 2 to 3 day intervals. A temperature of 105 and a leukocytosis of 10,200 followed the fifteenth injection (0.6 cc.). The animal was perfectly well the next day and remained so for a month following the nineteenth injection of 0.8 cc. The animal was in excellent condition and it would seem that a very high titer of immunity had been attained. So I injected 0.5 cc. of a potent suspension of poliomyelitis virus into the opposite frontal lobe (5-6-32). Nine days later the animal sickened. On the eleventh day he developed a fever of 104.3°F. and paralyses of the hind extremities and on the twelfth day paralysis of all extremities. He was chloroformed on the fifteenth day. One of the 4 control monkeys survived the intracerebral inoculation. There was difficulty in getting the virus into

the brain and there was considerable hemorrhage and escape of fluid through the wound. Possibly some of the virus was washed away.

From these observations it seems that I was able to protect monkeys against an intracerebral paralyzing dose of poliomyelitis virus by prolonged gradually increasing doses of phenolized cord tissue vaccine prepared after the Pasteur vaccine technique method. I was unable to protect them against a second intracerebral injection of the same virus. Autopsies showed typical brain and cord changes.

No evidence of allergy was demonstrated during the progress of the subcutaneous injections of vaccine or by intradermal testing at proper times with virus filtered through a Berkefeld M candle and heated at 57°C. for 30 minutes; 8 to 9 observations in 24 hours.

Pacific Coast Section.

University of California Hospital, December 12, 1934.

7795 C

Spectroscopic Determination of Gum Acacia in Blood. Rate of Disappearance in Normal Dogs.

S. A. PEOPLES AND N. M. PHATAK. (Introduced by C. D. Leake.)

From the Pharmacological Laboratory, University of California Medical School, San Francisco, California.

In the use of gum acacia in the treatment of nephrosis it is important to maintain a definite minimum concentration in the blood stream in order to derive satisfactory results. There is, therefore, need of a method for an accurate quantitative estimation of acacia on a sufficiently small sample of blood so that the test may be frequently repeated, especially when small children are involved. Acacia is a pentosan and all the methods now employed are based on the production of furfural by heating with dilute acid, steam distilling the furfural and determining it either by weighing as the phloroglucide[1] or by forming a color-complex between furfural and analine acetate.[2] Both methods require samples too large to make them practical for this purpose. Another method based on the Shaffer-Hartmann procedure for the determination of reducing sugars freed by hydrolysis of the Folin-Wu filtrate, as suggested in a personal communication from Dr. Hartmann, is some improvement. We have used this procedure on duplicate samples for comparison with a new spectroscopic method which we present herewith.

Our method is essentially a reversal of the Pettenkofer test for bile salts, with conditions so arranged as to make it quantitative for furfural. The details are as follows: 0.5 to 1.0 cc. of blood is precipitated according to the standard Folin-Wu technic. Five-tenths to 2 cc. of the filtrate, sufficient to contain 0.5 to 1.0 mg. of acacia, is pipetted into a 10 cc. volumetric flask with enough distilled water added to give a total volume of 2 cc.; and then 2 cc. of 80% sulfuric

[1] Meek, W. J., and Gasser, H. S., *Am. J. Physiol.*, 1918, **47**, 302.

[2] Youngburg, G. E., *J. Biol. Chem.*, 1927, **73**, 599.

acid are added. The contents are mixed and heated in a 100°C. water-bath for exactly 30 minutes (to assure complete hydrolysis) and are promptly cooled to room temperature in running water. Five cc. of 80% sulfuric acid and 1 cc. of 1% bile salts in 70% alcohol are added, mixed, and to bring out the color, the flask is placed in a 57°C. water bath for 20 minutes. The contents are immediately cooled to room temperature and after standing at least 10 minutes and not over 40 minutes are read in a spectrometer. The characteristic absorption band lies between E and b. A standard consisting of a dilute acacia solution is treated exactly as the Folin-Wu filtrate, 0.5 mg. and 1.5 mg. standards usually being sufficient. The color developed is always of the same tint and gives the same absorption band regardless of the concentration of the acacia. However, the intensity of color is accurately proportional to the amount of acacia in the sample. For satisfactory estimations the samples should be compared with standards of approximately the same concentration. With the 2 standards suggested, samples containing from 0.25 to 2 mg. may be satisfactorily compared. The conditions specified have been chosen to give maximal color production. This quantitative method is accurate within 5% for a 1 to 25 mg. sample but will detect 0.05 mg. qualitatively.

This method was used to determine the rate of disappearance of acacia from the blood stream in 5 normal dogs. Purified acacia in 20% solution was slowly injected in doses of 0.5 to 1.5 gm. per kg. and the acacia content of the blood was followed by our spectroscopic method and by that of Hartmann. The hemoglobin was

TABLE I.

Comparative estimates of acacia in blood samples by spectrometric and Hartmann methods.

Time Sample Was Taken After Acacia Injection 1 gm./kg.	Hemoglobin Gm./100 cc.	Blood Acacia, Gm./100 cc. Spectrometric Method	Hartmann Method
0	14.7	.00	.03
17 min.	13.0	.94	.85
35 "	12.7	.88	.92
1 hr.	11.8	.83	.83
2 "	11.5	.73	.72
4 "	12.0	.73	.80
8 "	12.6	.74	.71
12 "	12.3	.70	.62
24 "	11.6	.64	.52
36 "	11.1	.48	.45
48 "	10.1	.41	.36
72 "	10.9	.12	.13
96 "	12.6	trace	—
120 "	11.9	trace	—

determined colorimetrically using pure hematin as a standard. No other toxic symptoms than transient nausea and vomiting were noted. The results show a marked increase in blood volume in all cases, and indicate that several days are required for complete elimination of the acacia. The results of the spectroscopic method, herewith described, and of the Hartmann method are in good agreement as a whole, as shown in Table I. But it is to be noted that the Hartmann method is much more laborious, time-consuming, and requires a larger blood sample.

7796 C

Action of Dilaudid on the Gut.*

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From the California State Department of Penology, and the Pharmacological Laboratory, University of California Medical School.

David[1] has reported a comparative study of metabolic and other effects of dihydromorphinone hydrochloride ("Dilaudid" N.N.R.) and its parent substance, morphine. This new keto-derivative has a narcotic potency 5 to 10 times that of morphine, but apparently its use is not attended by freedom from the undesirable side-effects of morphine to quite the extent suggested by Alvarez.[2] Study of the relative addiction tendencies of the 2 drugs in human subjects is of great importance but will require competent clinical observation over an extended period because of the intrinsic difficulties in such a comparison. Meanwhile it is of interest to investigate the more easily measurable physiological side-actions of the new agent, as has been done for certain subjective and objective effects by David.

In regard to the effect of dilaudid on the gastro-enteric tract, David found no marked difference from morphine in the occurrence of nausea, vomiting, diarrhea, and constipation in student volunteers to whom there was administered subcutaneously a dose equivalent in narcotic efficacy to a therapeutic dose of morphine. Of 74 subjects receiving 0.01 to 0.04 mg. per kg. of dilaudid, 58% were nauseated, 20% vomited, 8% showed diarrhea and 30% con-

* Published with the permission of Mr. W. G. Walker, Chief, Division of Narcotic Enforcement, Department of Penology, State of California.

[1] David, N. A., *J. Am. Med. Assn.*, 1934, **103**, 474.

[2] Alvarez, W. C., *Proc. Staff Meet., Mayo Clinic*, 1932, **7**, 480.

stipation in comparison with 63% nausea, 27% vomiting, 3% diarrhea and 37% constiption respectively in 41 subjects receiving 0.14 to 0.22 mg./kg. of morphine sulphate.

Vomiting and nausea, which occur after the 2 drugs, are of central origin and it seems in humans that dilaudid does not vary much from morphine in the ratio of emetic dose to therapeutic dose. Leake[3] found that 10 mg./kg. of morphine sulphate causes emesis in dogs within 12 minutes, while we caused vomiting in only one of 4 dogs injected with 2 mg./kg. of dilaudid, although marked salivation and licking of chops were present in all. With subcutaneous dilaudid in dogs, the vomiting center may be too rapidly depressed, as with intravenous morphine, for emesis to occur regularly. That dilaudid definitely depresses the vomiting center after a slight initial stimulation, as demonstrated for morphine by Leake,[3] is shown by the fact that 1 mg./kg. of apomorphine HCl subcutaneously one hour after the administration of dilaudid failed to elicit any symptoms of vomiting whatsoever.

In order to ascertain whether or not further evidence might support David's observations on humans that dilaudid has essentially the same qualitative action on the gut as morphine, a series of experiments on animals were undertaken. As previously,[4] 3 pharmacological techniques were used. Macht's method indicated but little depression of peristalsis in the small intestine during the second hour following a subcutaneous injection of 0.1 mg./kg. of dilaudid, since the test meal traversed 53%±9% of the tract between pylorus and anus in 50 minutes as compared with 56%±6% in untreated animals. In rabbits, Eddy's technique demonstrated a depression of 75-100% in the number of rabbits of 10 defecating per hour, starting almost immediately after subcutaneous administration of 0.1 mg./kg. of dilaudid. Only one of 20 rabbits treated showed diarrhea, occurring after the fourth hour of observation. Results of these 2 tests indicate that the delay is chiefly in the colon and rectum, as is the case with morphine in man.[5] On strips of duodenum isolated from 6 different rabbits and suspended in oxygenated Locke's solution at 37°C., dilaudid was found to have slightly greater action than morphine in comparable therapeutic concentrations, particularly as shown by spastic contraction after washing out the drug. This suggests greater relative intensity of withdrawal symptoms, should dilaudid addiction occur. On this basis, also, dilaudid should be

[3] Leake, C. D., *J. Pharmacol. Exp. Therap.*, 1923, **20**, 359.

[4] Emerson, G. A., PROC. SOC. EXP. BIOL. AND MED., 1933, **31**, 278.

[5] Zehbe, M., *Ther. Mon.*, 1913, **27**, 406.

more helpful than morphine in preventing post-operative intestinal stasis.[6] The isolated gut shows no qualitative differences in reaction to morphine and dilaudid, and a morphinized gut shows no further depression with dilaudid and vice versa. In general, the findings of Uhlmann and Abelin[7] were confirmed in regard to the relative effects of various concentrations of morphine. While these observations are merely indicative of possible effects in patients, it may be expected that dilaudid will have much the same qualitative action on the gut as morphine but probably with less intensity in equivalent therapeutic dosage.

7797 C

Transformation of Hemolytic Streptococci.*

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From the Laboratory of Bacteriology and Experimental Pathology, Stanford University, California.

Two methods of typing hemolytic streptococci have been suggested by recent investigators: (a) the Lancefield method[1] based on the existence of at least 5 antigenically distinct type-specific "carbohydrates" in different human, veterinary and environmental strains, and (b) the Tillett-Garner-Madison technic,[2] based on the presence of at least 3 different type-specific fibrinolysins. Both methods assume that the selected diagnostic character is genetically stable.

To test this assumed stability an attempt was made to transform a typical antihuman fibrinolytic strain of *S. hemolyticus* into a non-fibrinolytic strain of apparent veterinary origin. The strain selected for this attempt was originally isolated by Lancefield from a case of scarlet fever. The strain (C203) is specifically lytic for human fibrin and contains only one type-specific carbohydrate.

To make the proposed transformation, 4 rabbits were injected intraperitoneally with doses varying from 2 to 20 cc. of a 24-hour

[6] Orr, T. G., *Ann. Surg.*, 1933, **98**, 835.

[7] Uhlmann, F., and Abelin, R., *Z. exp. Path. Therap.*, 1920, **21**, 58.

*Supported in part by the Rockefeller Fluid Research Fund of Stanford University School of Medicine.

[1] Lancefield, R. C., *J. Exp. Med.*, 1933, **57**, 571.

[2] Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485. Madison, R. R., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 641.

beef-heart-infusion broth culture. Three days later, the rabbit receiving 20 cc. was in a moribund condition. Blood was withdrawn by cardiac puncture and plated out on 2% rabbit-blood beef-heart-infusion agar. A single typical hemolytic colony on this plate, was used for the production of the broth culture for inoculation of the second group of 4 rabbits. This process was repeated for 22 successful animal passages.

Three times, during the 18 months this work was in progress, the virulence of the strain increased to approximately 700 times that of the original broth culture. In each case, however, the acquired virulence was almost completely lost before the next animal passage.

At varying stages of the transformation the recovered hemolytic colony was retitrated for the 5 Lancefield type-specific carbohydrates and for the Tillett-Garner antihuman fibrinolytic enzyme. Typical data thus obtained are recorded in Table I.

TABLE I. Effects of Repeated Rabbit Passage on Fibrinolytic Streptococci.

Five type-specific antistreptococcus serums were prepared from rabbits immunized against formalized type-cultures kindly furnished by Dr. Lancefield. Each antiserum gives specific precipitin reactions with 1:128 dilutions of the homologous Lancefield carbohydrate, and no demonstrable reaction with heterologous streptococcus extracts, the technic throughout being identical with that used by Lancefield.

In recording the data: ++++ represents precipitin reactions in dilutions as high as 1:128, +++ 1:32, ++ 1:8, and +1:2. (Type-specific antiserums B and D gave 0-reactions throughout the experiment.)

The antihuman fibrinolytic titres were determined by the Tillett-Garner plasma-clot technic; ++++ representing complete liquefaction of the normal human plasma-clot within 20 minutes after mixture with the 24-hour broth culture, +++ within 2 hours, ++ 6 hours, + 24 hours, and 0 no demonstrable softening by the end of 24 hours.

No. animal passages	"Carbohydrate" titer			Fibrinolytic titer
	Type A	Type C	Type E	
C203 before animal passage	++++	0	0	++++
After 1st rabbit passage	+++±	0	0	++++
" 4th " "	+++	0	0	
" 8th " "	++±	+++	0	+++
" 15th " "	0	0	+++	0
" 22nd " "	0	0	+++	0

From this table it is seen that both the Lancefield Type-A carbohydrate and the Tillett-Garner antihuman thrombolytic function disappeared quantitatively between the eighth and fifteenth animal passage. Two Lancefield carbohydrates were demonstrable in the eighth recovery strain. Both gave place to a third Lancefield carbohydrate (E) by the fifteenth animal passage. By this time, therefore, the original antihuman fibrinolytic strain of *S. hemolyticus*

had been successfully transformed and stabilized as a non-fibrinolytic strain of presumptive bovine origin.

That this is a justifiable interpretation of our data is shown by the almost perfect reversion of the 15th recovery strain to its original antihuman diagnostic specificities by the 7th subculture on routine culture medium. Typical stages in this reversion are recorded in Table II.

TABLE II. Qualitative and Quantitative Reversion on Routine Culture Medium.

A single hemolytic colony recovered from the 15th rabbit passage was transplanted at 2-week intervals on routine 2% rabbit-blood beef-heart-infusion agar. Titrations as in Table I.

No. of subculture	"Carbohydrate" titer			Fibrinolytic titer
	Type A	Type C	Type E	
C203 before animal passage	++++	0	0	++++
C203 after 15th rabbit passage	0	0	+++	0
3rd subculture of recovered strain	0	0	0	++
5th " " " "	++	0	0	+++
7th " " " "	+++±	0	0	+++
10th " " " "	++++	0	0	+++
15th " " " "	++++	0	0	+++±

7798 C

Susceptibility of "Hybrid" Fibrins to Streptococcus Fibrinolysins.*

R. R. MADISON. (Introduced by W. H. Manwaring.)

From the Laboratory of Bacteriology and Experimental Pathology, Stanford University, California.

Tillett and Garner[1] found that both of the human-rabbit heterologous fibrinogen-thrombin complexes are liquefied by the specific antihuman fibrinolysin formed or secreted by certain strains of *S. hemolyticus,* native rabbit fibrin being refractory to this specific bacterial lysin.

Quantitative differences, however, are demonstrable between the susceptibilities of the 2 Tillett-Garner hybrid fibrins. Fibrin formed by coagulating human-fibrinogen with rabbit-thrombin, for example, may require a 1:48 concentration of a given streptococcus filtrate to show demonstrable lysis. One-and-a-half times this lytic dose, or a

* Supported in part by the Rockefeller Fluid Research Fund of Stanford University School of Medicine.

1 Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

TABLE I. Liquefaction of Hybrid Fibrins by Antihuman Fibrinolysin.

Fibrinogens and thrombins were isolated from 11 animal species by the Tillett-Garner technic, and recombined in normal ratios to form homologous and heterologous fibrinogen-thrombin complexes. Each natural or artificial fibrin thus formed was tested against serial dilutions of selected fibrinolytic centrifugates, the technic being identical with that used by Tillett and Garner. The highest dilution of the centrifugate giving complete liquefaction of the fibrin-clot by the end of 2 hours was taken as the measure of its lytic susceptibility. The susceptibilities are recorded as percentages of normal human fibrin susceptibility.

Thrombin from:	Fibrinogen from:										
	Man	Monkey	Horse	Fowl	Rabbit	G.P.	Cow	Sheep	Dog	Cat	Swine
Man	**100%**	12	12	1	50	6	6	25	25	25	3
Rhesus monkey	100	8	0	0	8	0	0	0	0	0	1
Horse	100	8	0	0	0	0	0	0	0	0	0
Domestic fowl	90	2	0	0	0	0	0	0	0	0	0
Rabbit	75	4	0	0	0	0	0	0	0	0	0
Guinea pig	60	4	0	0	0	0	0	0	0	0	0
Cow	50	4	0	0	0	0	0	0	0	0	0
Sheep	50	4	0	0	0	0	0	0	0	0	0
Dog	25	0	0	0	0	0	0	0	0	0	0
Cat	25	0	0	0	0	0	0	0	0	0	0
Domestic swine	4	0	0	0	0	0	0	0	0	0	0

TABLE II. Liquefaction of Hybrid Fibrins by Antiveterinary Fibrinolysins.

The anti-horse fibrinolysin (upper half of table) was formed by a strain of *S. hemolyticus* originally isolated from a case of "horse strangles". The anti-swine strain (lower half of table) was originally isolated from a case of "hemorrhagic septicemia" in swine. This strain gives a slight cross-lysis with human fibrin. In the upper table normal horse-fibrin was taken as the 100% standard; in the lower table, normal swine-fibrin.

Thrombin from:	Fibrinogen from:										
	Man	Monkey	Horse	Fowl	Rabbit	G.P.	Cow	Sheep	Dog	Cat	Swine
Man	0	0	150	0	0	0	0	0	0	0	0
Rhesus monkey	0	0	100	0	0	0	0	0	0	0	0
Horse	100	25	**100%**	0	12	0	6	3	0	0	0
Domestic fowl	10	0	25	0	0	0	0	0	0	0	0
Rabbit	0	0	25	0	0	0	0	0	0	0	0
Guinea pig	0	0	25	0	0	0	0	0	0	0	0
Cow	0	0	25	0	0	0	0	0	0	0	0
Sheep	0	0	25	0	0	0	0	0	0	0	0
Dog	0	0	0	0	0	0	0	0	0	0	0
Cat	0	0	0	0	0	0	0	0	0	0	0
Domestic swine	0	0	50	0	0	0	0	0	0	0	0
Man	1	1	0	1	1	0	0	0	0	0	150
Rhesus monkey	0	0	0	0	0	0	0	0	0	0	100
Horse	0	0	0	0	0	0	0	0	0	0	200
Domestic fowl	0	0	0	0	0	0	0	0	0	0	100
Rabbit	0	0	0	0	0	0	0	0	0	0	25
Guinea pig	0	0	0	0	0	0	0	0	0	0	50
Cow	0	0	0	0	0	0	0	0	0	0	25
Sheep	0	0	0	0	0	0	0	0	0	0	100
Dog	0	0	0	0	0	0	0	0	0	0	50
Cat	0	0	0	0	0	0	0	0	0	0	12
Domestic swine	50	12	12	0	50	0	50	0	50	12	**100%**

1:32 concentration, may be required for a similar lysis of fibrin formed by coagulating rabbit-fibrinogen with human-thrombin. Since native human fibrin is dissolved by a 1:64 concentration of this filtrate, the relative susceptibilities of the 2 Tillett-Garner hybrid fibrins may be expressed as 75% and 50% of that of normal human fibrin.

Adopting this same percentage method of recording our experimental data, the relative susceptibilities of about 100 other hybrid fibrins are summarized in Table I.

From this table it is seen that both human-fibrinogen and human-thrombin are carriers of the normal human susceptibility to the Tillett-Garner streptococcus fibrinolysin. With certain human-veterinary hybrid fibrins an almost complete loss of the human susceptibility is noted. Both rhesus-fibrinogen and rhesus-thrombin are approximately 10% human in streptolytic susceptibility.

With the discovery[2] that certain veterinary streptococci are specifically lytic for lower animal fibrins, the above hybrid fibrins were retested with 2 antiveterinary fibrinolysins. Data thus obtained are recorded in Table II.

In these tests, also, both the fibrinogen and thrombin of the susceptible animals (horse, swine) are species-specific in their relationships to streptofibrinolysins. There is here, also, an apparent denaturation of the susceptible component in certain hybrid fibrins. Three hybrid fibrins show fibrinolytic hypersusceptibility.

7799 C

Attempt to Modify Hereditary Chromogenic Genes by Heterophile Antimelanin Immunization.*

CARL-GUSTAF TILLMAN. (Introduced by W. H. Manwaring and C. H. Danforth.)

From the Laboratory of Bacteriology and Experimental Pathology and the Department of Anatomy, Stanford University, California.

The demonstration by Adant[1] that beef-choroid melanin is antigenic for albino rabbits and that the resulting antiserums give com-

[2] Madison, R. R., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 444.

* Work supported in part by the Rockefeller Fluid Research Fund of Stanford University School of Medicine.

[1] Adant, A., *Compt. rend. Soc. Biol.*, 1932, **111**, 241.

plement-deviation reactions with rabbit-melanin, has suggested the possibility of modifying hereditary chromogenic functions by heterophile antimelanin immunization.

To test this possibility, a series of 12 pedigreed female albino rabbits were given 6 subcutaneous, 6 intraperitoneal, followed by 6 intravenous injections with native beef-melanin, at 5-day time intervals, the technic identical with that used by Adant. About 4 days after the final immunizing dose, the presumably melanin-immune albino females were mated with a pedigreed heterozygous black. Subcutaneous melanin injections were resumed 3 to 6 days later and continued at weekly intervals throughout pregnancy.

Eight litters, born to these presumably melanin-immune albino mothers, and 4 control litters born to non-immune albino females, are summarized in Table I.

TABLE I.

Litter number	Pigment Distribution			Non-immune Controls		
	Black	Gray	White	Black	Gray	White
1	4	0	4	3	0	3
2	4	0	3	4	0	4
3	2	0	1	4	0	3
4	1	1	1	4	0	4
5	3	0	6	—	—	—
6	6	0	4	—	—	—
7	4	0	2	—	—	—
8	2	0	9	—	—	—
Total	45%	2%	53%	52%	0%	48%

Within the limits of the experimental error, no modification of hereditary chromogenic functions is demonstrable by this method of heterophile immunization.

7800 C

A Test for Filtrable Forms of M. Tuberculosis Using Ultrafilters.

EVELYN S. LEWIS, MERLE RUCKMAN AND FLORA JAMES. (Introduced by A. P. Krueger.)

From the Department of Bacteriology, University of California, Berkeley, Calif.

A vast amount of work has been done which apparently supports the theory that a filtrable stage exists in a life cycle of *M. tuberculosis*. It should be noted that the outstanding criticism of all such work lies in the fact that in each case use has been made of Cham-

berland or Berkefeld filter candles which are difficult to standardize inasmuch as they involve factors such as electrical charge of the particle, medium in which the particle is suspended, as well as pore size. Until a filtration process can be devised which would permit exact duplication at any time and which could be standardized and controlled to retain a given size of particle, proof would be inconclusive that a filtrable form of *M. tuberculosis* exists.

For this reason it was felt that more significant data could be obtained with the ultrafilters of Krueger and Ritter[1] in which such factors as filter charge and adsorption of filtrate play practically no part, the membranes actually operating on the principle of a sieve. By carefully controlled experiments they not only graded the collodion membranes of varying pore sizes on the basis of permeability to colloidal particles of known size but also determined the degree of negative pressure which could be used in each case without causing distortion of the membrane. It was found that by using a negative pressure of 20 cm. a 4.5% collodion membrane (with pore diameter 40μμ) retained all known bacteria.

The following filtration experiments, therefore, were carried out on 4.5% and 5.0% collodion membranes. The strains of tubercle bacilli used had been freshly isolated from sputum and cultivated both on Sweaney's egg-glycerol medium and on glycerine veal broth. Microscopic examination of the culture on Sweaney's medium after 6 weeks' incubation revealed many smoothly staining acid-fast rods with few granular forms while in the latter case the acid-fast granules predominated after 3 months' incubation.

The criterion for complete retention by this method as tested upon filtrates of the centrifugalized suspensions of the organisms was considered to be: 1. Lack of infection of guinea pigs as determined by tuberculin tests and absence of lesions and organisms, acid-fast or otherwise, in the spleen, liver, lungs and lymph glands of the test animals following subcutaneous inoculation of the filtrate in the inguinal region.

2. The absence of any microscopic forms, acid-fast or otherwise.

3. The absence of growth on Sweaney's egg-glycerol medium.

The controls for these experiments consisted of the following tests on the original saline suspension before filtration:

1. The failure of *B. prodigiosus* to pass through the collodion membrane when filtered simultaneously with the saline suspension of *M. tuberculosis.*

2. The experimental infection of tuberculosis-free guinea pigs;

[1] Krueger, A. P., and Ritter, R. C., *J. Gen. Physiol.*, 1929, **13**, 409.

the test animals were found to give positive tuberculin tests after intervals of 4 and 8 weeks. Recovery of acid-fast organisms was made from the liver, spleen and lymph nodes.

3. The cultivation of the organisms on Sweaney's egg-glycerol medium.

Three separate series of tests were made on each type of membrane. Two guinea pigs were used for the controls and 2 for each test. Each animal was previously found to be tuberculosis-free as determined by the tuberculin test. An even emulsion of the organism was obtained by thoroughly grinding the growth as washed from the solid media in physiological saline.

Our results show that the ultrafiltrates did not contain infectious particles capable of producing: (1) a positive tuberculin reaction; (2) anatomical evidences of the infection in experimental animals or growth of tubercle bacilli on appropriate media for their cultivation. It may be concluded, therefore, that the cultures tested did not develop "filterable forms" smaller than 40 μμ in diameter.

7801

Colloidal Calcium Phosphate of Blood and Calcium Partition in Serum.

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From the Division of Biochemistry, University of California Medical School, Berkeley.

It has now been thoroughly shown that under certain conditions the inorganic phosphate of the serum becomes incompletely diffusible.[1] A logical explanation of this phenomenon is that the nondiffusible phosphate which is formed is combined with some of the serum calcium in a compound which is in a colloidal state. As yet, no direct evidence is available as to the exact composition of this colloidal calcium phosphate.

On the basis of the view which has recently received strong support from the work of McLean and Hastings,[2] namely, that there is an equilibrium between the ionized and the protein bound calcium in

[1] See Schmidt, C. L. A., and Greenberg, D. M., *Physiol. Rev.*, in press, for literature.

[2] McLean, F. C., and Hastings, A. B., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 529; *J. Biol. Chem.*, 1934, **107**, 337.

serum which is governed by the mass law, it appears possible to develop a relationship which will quantitatively account for the partition of the serum calcium and one which will also yield a plausible value for the composition of the colloidal calcium phosphate. In the following treatment it is assumed that the diffusible calcium is virtually equivalent to the ionized calcium of the serum. This opinion also is supported by the work of McLean and Hastings.

For the present purpose, the mass law relationship may be conveniently written in the form,

$$\frac{P}{CaP} = \frac{1}{A} + \frac{B}{Ca_D^{++}} \qquad (1)$$

where P represents the protein, CaP the calcium protein complex, and Ca_D^{++} the diffusible calcium. A and B are constants whose significance is given below. The equation as given has the advantage that if the observed data obey the mass law, then a straight line is obtained on plotting the values of $\frac{P}{CaP}$ against $\frac{1}{Ca_D^{++}}$. The value of the constant A is given by the reciprocal of the point of intersection of the straight line on the P/CaP axis. It has the significance of being the maximum amount of calcium which can be bound by each unit weight of protein. The slope of the straight line gives the value of the constant B. This term consists of the equilibrium constant divided by A.

The application of this theory as applied to drawn beef blood serum, which has been augmented with varying amounts of calcium, is illustrated by the data given in Table I. The results of 4 experimental series on blood taken from different animals are given in the table.

The most satisfactory results were obtained when the colloidal calcium phosphate was assigned a composition of $Ca_3(PO_4)_2$. The amount of non-diffusible calcium present in this form in any sample was calculated from the non-diffusible inorganic phosphate content of the serum. The value so obtained, subtracted from the total non-diffusible calcium, yields the protein-bound calcium.

The values of the protein-bound calcium determined in this manner were plotted against the diffusible calcium values according to the method which has been indicated. The plot yielded the numerical equation,

$$\frac{P}{CaP} = 0.51 + \frac{3.5}{Ca_D^{++}} \qquad (2)$$

where the protein content is expressed in grams and the calcium

TABLE I.
Calcium and Inorganic Phosphate Partition in Beef Serum Augmented with Calcium.

Series	Protein %	Calcium			Inorganic Phosphate			$Ca_3(PO_4)_2$ calcium	Protein Bound Calcium	
		Serum	Diffusible mg. %	Non-diffusible	Serum	Diffusible mg. % of P	Non-diffusible		Experimental	Calculated
A-1	7.35	11.2	4.8	6.4	5.55	5.55	0	0	6.4	5.9
A-2	7.3	15.6	6.6	9.0	5.55	4.5	1.05	2.0	7.0	7.0
A-3	7.3	20.0	8.8	11.2	5.5	3.8	1.7	3.3	7.9	8.0
A-4	7.2	29.9	12.9	17.0	5.5	1.4	4.1	7.9	9.1	9.2
B-1	8.6	11.3	4.0	7.3	6.6	6.1	0.5	0.9	6.4	6.2
B-2	8.5	19.4	8.6	10.8	6.55	2.75	3.8	7.3		
B-3	8.45	29.3	10.9	18.4	6.5	1.8	4.7	9.1	9.3	10.1
B-4	8.35	38.3	15.3	23.0	6.4	0	6.4	12.4	10.6	11.2
B-5	8.30	47.1	21.5	25.6	6.4	0	6.4	12.4	13.2	12.4
C-1	6.75	11.7	6.0	5.7	7.5	7.5	0	0	5.7	6.2
C-2	6.65	20.4	7.3	13.1	7.4	4.0	3.4	6.6	6.5	6.7
C-3	6.60	29.2	11.2	18.0	7.3	1.8	5.5	10.6	7.4	8.0
C-4	6.55	37.7	17.2	20.5	7.3	1.3	6.0	11.5	9.0	9.2
C-5	6.50	46.4	24.0	22.5	7.2	0.7	6.5	12.5	10.0	9.9
D-1	6.9	9.3	4.0	5.3	7.7	7.7	0	0	5.3	5.0
D-2	6.9	14.1	5.5	8.6	7.7	6.25	1.45	2.8	5.8	6.0
D-3	6.85	17.9	6.6	11.3	7.65	5.0	2.65	5.1	6.2	6.6
D-4	6.8	22.3	7.8	14.5	7.6	3.85	3.75	7.2	7.3	7.1
D-5	6.8	26.6	10.7	15.9	7.55	3.25	4.3	8.3	7.6	8.1

values in milligrams per 100 cc. of serum. From the A value in equation (2) there is obtained the information that 1.96 mg. of calcium should be the maximum amount capable of being bound per

gram of beef serum protein. This result would yield a value of 14 mg. % for the protein-bound non-diffusible calcium in a serum with a protein concentration of 7.2%. This figure harmonizes with a previous deduction of Greenberg and Gunther[3] that with increasing calcium ion concentration the protein-bound calcium increases only by a moderate amount. From the value of B in equation (2) the equilibrium constant of the mass law relationship is found to have the value of $10^{-2.75}$ when expressed in units of mols per liter. This is of the same order of magnitude as the value of the equilibrium constant found by McLean and Hastings for human blood serum.

The degree to which the experimental data fit the equation given is indicated by the results in the last column of Table I, under the heading, "Protein Bound Calcium, Calculated." From a comparison of the figures calculated according to equation (2), which are given in this column, with the experimentally obtained values, it is seen that the agreement is satisfactory. The results which are presented appear to favor the viewpoint that the colloidal calcium phosphate found in blood serum has the composition of the tertiary salt and that the equilibrium between the protein-bound and the ionized calcium conforms to the mass law.

7802 P

Kidney Glomerulus of Hypertension Produced Experimentally by Pituitary Excess.

RAYMOND F. BLOUNT. (Introduced by C. M. Jackson.)

From the Institute of Anatomy, University of Minnesota.

That the thickened basement membrane of glomerular capillaries in hypertension is primarily due to the hypertension itself has not been definitely shown and the suggested relationship of the pituitary to hypertension has been based upon insufficient clinical evidence. The present experiments have produced in the amphibian a condition paralleling hypertension in the mammal with vasoconstriction, slowed heart rate, thickened ventricular wall and edema (Blount[1]). This has been accomplished by the transplantation of additional pituitary anlagen in the embryo (Blount[2]) which develop and give

[3] Greenberg, D. M., and Gunther, L., *J. Biol. Chem.*, 1930, **85**, 491.

[1] Blount, R. F., *Anat. Rec.*, 1933, **55**, Suppl. 7.

[2] Blount, R. F., *J. Exp. Zool.*, 1932, **63**, 113.

a constant excess of hormone secretion bringing about the results described. The constant secretion is indicated by pigmentation changes and vasoconstriction which are maintained. Since this takes place during the development of the structures involved there is an adjustment which tends to establish a balance at the high pituitary level.

The glomerulus of the mesonephros in all the 15 triple pituitary animals examined shows certain changes which are characteristic of the hypertensive glomerulus in man. As shown by either Mallory's stain or by azocarmine the basement membrane of the capillary tuft of the normal animal is roughly 1 to 2 micra thick while that of the triple pituitary animals may exceed 8 micra. This membrane may exhibit lighter areas which appear to be small cracks or pores and is similar to that of hypertension in man especially when associated with lipoid nephrosis. Many of the capillaries are closed. The glomerulus is small in respect to the capsule which it does not fill to the normal degree. There are accompanying changes in the tubules which are associated with glomeruli that are most affected. These changes are progressive with age. The degree of involvement is directly proportional to the amount of pituitary excess as measured by the degree of response in such characters as pigment increase.

This is the first time the basement membrane change has been produced experimentally and shows that here hypertension is the cause and not the result of the thickening as it develops early. In these animals the hypertensive glomerulus is definitely associated with pituitary excess. An attempt is being made to produce similar changes in the metanephros of the mammal by the administration of posterior pituitary hormone.

7803 P

Vitamin C Content of Tissues of Laboratory Animals Under Various Pathological Conditions.

E. HARDE AND HELEN RIVKIN BENJAMIN. (Introduced by W. H. Park.)

From the Department of Health, Research Laboratory and Department of Bacteriology, New York University.

We have studied the vitamin C content of the adrenals of laboratory animals injected with bacterial toxins, or viruses, or dying from spontaneous infections, using a silver nitrate solution or the

indicator 2:6 dichlorophenol-indophenol (technique of Birch, Harris and Ray[1]). By this latter method, we have also examined the tissues of guinea pigs apparently completely recovered from sub-lethal injections of diphtheria or tetanus toxins and fed excess vitamin C.

Determination by silver nitrate. Having found the adrenals of guinea pigs dying of diphtheria toxin did not reduce silver nitrate, while those of the control animals were rapidly blackened,[2] we applied this method to the adrenals of animals fed a complete diet showing various pathological conditions with the following results.*

A positive reaction was found in rabies-fixed virus (4 rabbits); in infection with human tubercle bacilli of moderate virulence (3 guinea pigs), killed 2 months after inoculation, showing slight lesions; and after injections of toxin of dysentery of dysentery bacilli (3 rabbits) dead in 18 to 48 hours.

A diminution, more or less marked, of the reducing power of the adrenals, especially the cortex, occurred after a lethal injection of tetanus toxin (5 guinea pigs); of dysentery toxin (4 mice, dying in 3 to 5 days); in mouse typhoid (4 mice infected *per os* and dying in 4 to 6 days); and after repeated injections of histamine-hydrochloride† (4 guinea pigs and 3 mice).

In yellow fever the reactions gave irregular results in mice dying after intracerebral inoculations. In one case rapid blackening occurred; in 2 others the reaction was incomplete.

Determination by 2,6-dichlorophenolindophenol titrations. By this method the tissues of supposedly normal guinea pigs (200-300 gm.) were examined. These animals were bought in the open market and their previous diet was unknown. The ascorbic acid content of their organs varied greatly. The figures are given in mg. per gm., adrenals 0.27-0.66, liver, 0.05-0.12. Variations were also noted in the tissues of guinea pigs fed carrots, hay and oats for 10 days, adrenals 0.15-0.30, liver 0.02-0.04.

Eleven animals dying from diphtheria toxin gave the following values: adrenals 0.06-0.15, liver 0.03-0.06.

Seven guinea pigs having recovered from sub-lethal injections of diphtheria toxin, were fed a diet containing excess spinach or cab-

[1] Birch, T. W., Harris, L. I., and Ray, S. N., *Biochem. J.*, 1933, **27**, 590.

[2] Harde, E., *C. R. de l'Acad. des Sc.*, 1934, **199**, 618.

* This work was done at the Pasteur Institute, Paris, during the spring and summer months. The animals were fed abundant green, hay, oats or bread.

† Adrenals and gastrointestinal tracts are congested and occasionally there are gastric ulcers and hemorrhages after these injections.

bage. High values of Vitamin C were found in the tissues of 2 of these animals which had shown but a slight reaction, adrenals 0.77-0.68, liver 0.24-0.11, pancreas 0.27-0.20. In 5 other guinea pigs having shown a severe reaction a lowered content was found, adrenals, 0.13-0.47, liver 0.04-0.15.

Three guinea pigs dying from a spontaneous infection, (marked lesions in lungs and liver) gave low values, even though the diet contained excess vitamin C, adrenals 0.08-0.10, liver 0.07-0.05.

For purposes of record we may also note the ascorbic acid content found in the tissues of 2 rhesus monkeys infected with poliomyelitis‡ (paralyzed and killed), adrenals 0.45 and 0.23, liver, 0.18 and 0.11. With silver nitrate the reaction was slow and incomplete.

Thus, in the tissues of laboratory animals we have found a reduction in the ascorbic acid content in many infections and intoxications.

Recently Yavorsky, Almaden and King[3] studied quantitatively ascorbic acid in tissues of humans having died of various causes. These authors note a diminution of vitamin C in the majority of cases of general infection.

Worringer and Sala[4] reported that, among other infections, diphtheria gave rise to infantile scurvy. They also cite 4 cases of whooping cough in children, followed by scurvy, and cured by the juice of fresh fruits.

7804 P

Action of Thallium in Experimental Animals.*

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A number of investigators of thallium poisoning have attributed some of the changes in poisoned animals to disturbance in function of the endocrine glands and the sympathetic nervous system. In support of this relationship Buschke and his associates[1] have re-

‡ These were kindly supplied to us by Dr. Brodie.

[3] Yavorsky, M., Almaden, P., and King, C. G., *J. Biol. Chem.*, 1934, **106**, 525.

[4] Worringer, J., and Sala, A., *Rev. Franc. de Pediat.*, 1928, **33**, 809.

* Supported in part by a grant from the Rockefeller Fluid Research Fund.

[1] Buschke, A., and Berman, L., *Münch. Med. Wchnschr.*, 1927, **74**, 969. Buschke, A., Zondek, B., and Berman, L., *Klin. Wchnschr.*, 1927, **6**, 683. Bickel, L., and Buschke, A., *Klin. Wchnschr.*, 1932, **11**, 679.

ported cessation of the estrual cycle in mice after thallium acetate administration. Wu and Hu[2] reported a temporary fall in the basal metabolic rate of rats after single injections of thallium acetate. Further study of the effect of thallium on the estrual cycle in rats and the basal metabolic rate in guinea pigs was undertaken by us in an attempt to demonstrate changes which might be due to alteration of endocrine function.

Thallium acetate was injected subcutaneously into 20 female white rats in doses ranging from 0.2 to 5 mg. of the metal per kg. body weight 3 times per week over periods up to 9 weeks in length. The animals given the larger amounts showed signs of thallium intoxication, and those receiving doses of 5 mg. per kg. died about 9 days after the first injection. There were no changes in the estrual cycle as measured by the vaginal smear method of Long and Evans.[3] Histological studies of the vagina, uterus, and ovaries showed no constant differences from control organs.

Subcutaneous injection of the same compound into 4 young adult male guinea pigs in doses of 3 mg. thallium per kg. body weight 3 times per week produced no demonstrable changes in basal metabolic rate over periods of 3 to 6 months. The determinations were made from measurements of oxygen consumption during intervals of inactivity after preliminary 16-hour starvation periods. Injections of 6 mg. thallium per kg. 3 times per week in the same manner produced death of 6 male guinea pigs in about 8 days. Metabolism determinations on these animals showed no deviation from the control level until within 24 hours of death when there was a pronounced decrease in oxygen consumption associated with signs of severe intoxication: apathy, salivation, diarrhea, loosening of the hair, and a fall in body temperature to as low as 31°C. The rate of oxygen consumption in one animal fell to a point 75% below the control level, and that of another to 62% below the average control value. In view of the absence of any change in oxygen consumption until shortly before death, and the extent of the decrease when it occurred, it is probable that the effect is not a specific result of the action of thallium upon the endocrine glands. Kaiwa[4] and others have reported decreases in basal metabolism of 25 to 35% in completely thyroidectomized animals, and decreases only up to

[2] Wu, J., and Hu, C., PROC. SOC. EXP. BIOL. AND MED., 1930, **27**, 251.

[3] Long, J. A., and Evans, H. N., *Univ. of Calif. Publication*, 1922, vi.

[4] Kaiwa, T., *Tohoku J. Exp. Med.*, 1932, **19**, 96.

40% in rabbits after total removal of the thyroid and both adrenal glands.

Conclusion. White rats given large or small quantities of thallium over periods up to 9 weeks in length showed no abnormality of the estrual cycle or of the reproductive organs. The basal metabolic rate of guinea pigs did not change during administration of small or large doses of thallium, except for a rapid decrease shortly preceding death. These findings fail to support previous claims that thallium intoxication is characterized by alteration of endocrine function.

7805 C

Hemorrhagic Changes in Suprarenal Cortex of Adult Rats Following Pituitarectomy.

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Smith and many other investigators have demonstrated that removal of the pituitary gland in tadpoles and rats is followed by atrophy of the suprarenal cortex,[1-8] as well as atrophy of the gonads, thyroid and parathyroid. Similar changes have been described in the rabbit[9, 10] and dog.[11]* In humans, in conditions associated with hypofunction of the pituitary and in Simmond's disease the adrenal

[1] Smith, P. E., *Am. Anat. Mem.*, 1920, **11**, 151.

[2] Atwell, W. J., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 621.

[3] Smith, P. E., *Anat. Rec.*, 1926, **32**, 221.

[4] Smith, P. E., *J. Am. Med. Assn.*, 1927, **88**, 158.

[5] Smith, P. E., *Am. J. Anat.*, 1930, **45**, 205,

[6] Richter, C. P., and Wislocki, G. B., *Am. J. Physiol.*, 1930, **95**, 481.

[7] Evans, H. K., Meyer, M. E., Simpson, *et al., Mem. Univ. Cal.*, 1933, **2**.

[8] Collip, J. B., Selye, H., and Thompson, D., *Nature*, 1933, **131**, 56.

[9] Ikeda, M., *Jap. J. Obst. and Gynecol.*, 1932, **15**, 213.

[10] Kusonoki, J., *Folio Endocrinol.*, Japan, 1927, **3**, 34.

[11] Houssay, B. A., and Sammartino, R., *Compt. rend. Soc. de Biol.*, 1933, **114**, 717.

* The atrophy of the suprarenal cortex in pituitarectomized rats may be repaired by daily homotransplants of pituitary gland,[5] by injection of potent extracts of growth hormone,[7] or by the adrenotropic hormone.[16] The administration of cortin in pituitarectomized rats does not prevent or repair this atrophy of the cortex.[17]

[16] Anderson, E. M., Thomson, D. L., and Collip, J. B., *Lancet*, 1933, **225**, 347.

[17] Shumacker, H. B., and Firor, W. M., *Endocrin.*, 1934, **18**, 676.

glands are atrophic.[12-15] The regressive changes in the suprarenal glands of immature rats are noted as early as the 7th day, are pronounced by the 15th day and reach their maximum by the 30th day, though the cortex may continue to decrease in size after this (Smith[5]).

In studies on the relation of the pituitary gland to resistance[18] in adult rats, we noted the early changes preceding this involutional atrophy of the suprarenal cortex.

Method. The rats were adults, 3 to 4 months old, of a uniform stock raised in our laboratory. The observations were made on 20 pituitarectomized rats that died spontaneously in the course of other experiments within 4 to 10 days after operation. Control observations were made on 15 rats in which large fragments of anterior lobe remained and on 20 operated controls. In a second group, 10 rats were pituitarectomized and 2 rats were killed on the 4th, 5th, 6th, 7th, and 9th day after operation. The suprarenals were immediately removed and fixed in Bouin's fluid.

Results. From 4 to 10 days after pituitarectomy the suprarenal gland showed striking changes. Hemorrhages appeared in the reticular zone of the cortex. Marked engorgement of the capillaries was noted and the cells showed evidence of vacuolization and cloudy swelling. The nuclei were often vacuolated. In other instances in which the hemorrhage was less prominent or absent the cells appeared shrunken and the cytoplasm diminished in amount. In many instances large macrophages filled with iron pigment were observed in the interstitial tissue of reticular zone and between the cortex and medulla. When sections were stained by the Turnbull method a broad blue zone of iron pigment was seen in the inner layers of the cortex. The hemorrhagic changes were observed as early as the 3rd day and as late as the 21st day after operation but apparently occur with greatest frequency about 7 to 9 days after pituitarectomy. It is probable that the hemorrhagic changes in the suprarenal cortex precede actual shrinkage and atrophy of the cells of the cortex. The presence of cortical hemorrhages was noted in rats that showed no evidence of infection and in which no other procedure had been done.

The daily administration subcutaneously of a crude saline emulsion of fresh anterior lobes of ox pituitary during a period of 2

[12] Falta, W., *Die Erkrankungen der Blutdrüsen*, Springer, Berlin, 1913.

[13] Cushing, H., and Davidoff, L. M., *Monograph, Rockefeller Inst.*, 1927, **22**, 1.

[14] Simmonds, M., *Deutsche med. Wchnschr.*, 1919, **45**, 487.

[15] Erdheim, J., *Beitrage z. path. Anat. u. z. allg. Path.*, 1916, **62**, 302.

[18] Perla, D., in press.

weeks prior to pituitarectomy caused marked enlargement of the suprarenal glands (as well as increasing the size of the thyroid and gonads, and the rate of growth). After operation the hemorrhagic changes were definitely delayed.

These observations are of significance in the interpretation of the spontaneous involution of the suprarenal cortex which occurs during the first month of life in human beings. The extraordinary degenerative changes which take place in the suprarenal cortex of infants soon after birth were described by Thomas,[19] Elliott and Armour,[20] Kern,[21] Landau,[22] and in great detail by Lewis and Pappenheimer.[23] These observers noted that during the latter half of pregnancy the suprarenal is unusually large. This enlargement is due to a hypertrophy of the cortex. Shortly after birth (during the second week of extra-uterine life) according to Lewis and Pappenheimer a massive degeneration of the 2 inner layers of the cortex occurs, associated with more or less hemorrhage and fatty change of the cells. The degeneration is fully established by the end of the second week and during the next 5 weeks rapid removal of the broken down cells occurs, leaving a relatively thin glomerular layer that collapses on the medulla. Regeneration does not occur up to the third year.

The physiological significance of this degenerative involution was not clear. Marine, Lowe and Cipra noted that there is a rise in heat production in infants during the second week of life and this is associated with other signs of increased tissue activity. They believed that the rise in heat production beginning the second week of life may be related to the normal destruction of the reticular layer of the suprarenal cortex. With the newer knowledge of the interrelation of the pituitary and suprarenal glands, it would appear as Marine[24] suggests, that the suprarenal involution in infants was due to the sudden withdrawal of the maternal source of adrenotropic substance at birth. It is striking that the involution of the suprarenal following pituitarectomy in adult rats is preceded by hemorrhagic changes within one week of operation, analogous to those observed 8 days after birth in humans. It is obvious that the withdrawal of the adrenotropic hormone of the pituitary is responsible for the changes in the rat.

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[19] Thomas, E., *Ziegler's Beitr.*, 1911, **50**, 283.

[20] Elliott, T., and Armour, R., *J. Path. and Bact.*, 1911, **15**, 481.

[21] Kern, H., *Deutsch. med. Wchschr.*, 1911, **37**, 981.

[22] Landau, M., *Die Nebennierenrinde*, Jena, 1915, G. Fischer.

[23] Lewis, R. W., and Pappenheimer, A. M., *J. Med. Res.*, 1916, **34**, 81.

[24] Marine, D., *J. Am. Med. Assn.*, 1935, in press.

Summary. Within the first 2 weeks following pituitarectomy in adult albino rats hemorrhagic changes are observed in the cortex of the suprarenal which precede the atrophy of the gland. The similarity to the changes observed soon after birth in humans is pointed out.

By inference this observation tends to strengthen the hypothesis that the severe hemorrhagic involutional changes noted in early extra-uterine life may be due to the withdrawal of the maternal adrenotropic hormone of the anterior lobe.

7806 P

Enzymatic Efficiency in Malignancy.*

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Recently we have begun a detailed investigation of enzymatic efficiency of avitaminosis.[1] Being at the same time engaged in a problem on the relationship of vitamin therapy to regression of tumors in the albino rat, we had access to considerable numbers of tumor-bearing animals and had available the necessary materials and equipment to study the enzymatic efficiency of malignancy.

We found our Wistar stock of animals rather resistant to the Flexner-Jobling carcinoma; consequently, the greater portion of our work was done on animals receiving another type of transplantable tumor, in which we had from 60 to 85% takes, provided the tumor used for implantation was not too necrotic. The tumor used was furnished by Dr. Francis Carter Wood of New York City, which was designated as Walker Carcino-Sarcoma No. 256.

The experiments were conducted in pairs, and for every animal with an implanted tumor, a litter-mate control of the same sex was used, which was restricted to the same plane of nutrition as the tumor-bearing animal. The tumors were measured with a caliper in length and width, and in some cases in depth. Forty-two groups were studied in the case of most enzymes. The blood serum phos-

* Research Paper No. 320, Journal Series, University of Arkansas, Fayetteville. Supported by a fund from the International Cancer Research Foundation.

[1] Sure, B., Kik, M. C., and Buchanan, K. S., *J. Biol. Chem.*, 1935, **108**, 11.

phatase was included later in the investigation and was studied in 26 groups. The tumors ranged from 15 to 73 mm. in length and from 10 to 65 mm. in width. The age of the tumors of the implanted animals, the blood and tissue enzymes of which were studied, ranged from 3 to 5 weeks in most cases, but some individuals were allowed to proceed to the stage of pronounced ulceration and these tumors were about 7 weeks old at the time the animals were sacrificed. The tumors were embedded in formalin and examined microscopically by Drs. Thatcher and De Groat in Little Rock. The external observations showed cases varying from non-ulceration and slight ulceration to pronounced ulceration with pus oozing from the exterior. On microscopic examination there were present degeneration, necrosis and inflammation varying from one-fourth to three-fourths of the entire tumor. Ten different enzymes were investigated on the same animals, and Table I gives a general summary of these results, the details of which will appear elsewhere.

TABLE I.

% decrease in concentration of various blood and tissue enzymes of tumor-bearing albino rats compared with litter-mate controls of the same sex restricted to the same diet and same plane of nutrition.

Enzyme		Enzyme	
Blood Serum Amylase	48.5	Blood Serum Phosphatase	11.1
Blood Serum Esterase	47.0	Hepatic Lipase	8.7
Trypsin	39.0	Pancreatic Lipase	8.3
Hepatic Esterase	16.5	Pancreatic Esterase	7.3
Pancreatic Amylase	13.5	Erepsin	5.2

It is apparent that the most marked changes were found in the blood serum amylase, blood serum esterase and in trypsin. Small but definite changes were detected in hepatic esterase, pancreatic amylase, and blood serum phosphatase. The decrease in hepatic and pancreatic lipase, in pancreatic esterase and in erepsin are too small to be considered of any significance.

It is interesting to note that in 9 groups of animals implanted with the Flexner-Jobling Carcinoma practically the same effect of pronounced decreases in the blood serum amylase and esterase were found as in the 42 groups of animals implanted with the Walker Carcino-Sarcoma No. 256 furnished by Dr. Francis C. Wood.

We are continuing our investigations with other enzymes and other tumors.

7807 C

Negative Results of Treatment of Chronic Myelogenous Leukemia as a Deficiency Disease.*

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There is no obvious reason for supposing that chronic myelogenous leukemia is a dietary deficiency disease. Dietary inadequacy or abnormalities of the gastrointestinal tract, almost invariably present in the deficiency group of anemias including pernicious anemia, apparently rarely occur in chronic myelogenous leukemia. On the other hand, the abnormal proliferation of primitive white blood cells in the bone marrow of myelogenous leukemia might conceivably have a physiological analogy in the maturation arrest of the primitive red blood cells crowding the bone marrow of pernicious anemia in relapse. Although gastric anacidity is only occasionally present in myelogenous leukemia, the simultaneous advent of 2 patients with chronic myelogenous leukemia, one of whom was in a leukopenic phase and both of whom had gastric achlorhydria, provided the stimulus for performing the observations described below.

An attempt was first made to supplant an entirely hypothetical defect of the gastrointestinal secretions of the 2 patients by administering alone, or in combination with other tissues, preparations of certain organs known to be effective in pernicious anemia. The daily intramuscular injection for 20 days of liver extract 343 N.N.R., derived from 25 gm. of liver, in each of the 2 patients mentioned above, was without effect. Next, the daily administration for 20 days of combined desiccated preparations derived from 100 gm. of gastric mucosa, from 250 gm. of the mucosa of the small intestine, and from 50 gm. of the pancreas of the hog, respectively, was carried out. In one of these patients the daily administration for 11 days of about 150 gm. of the mixed fresh tissue (except for the head, skin, and extremities) of newborn rabbits was then instituted in an attempt to supply a "shotgun" preparation of normal animal tissues and their metabolic products. All of these procedures were entirely without effect upon the clinical and hematological features of the disease. Similar negative results were obtained by

* The expenses of the present investigation were borne in part by grants from the J. K. Lilly gift to the Medical School of Harvard University.

Forkner and Taylor[1] with the oral administration of whole liver and calf bone marrow and the injection of aqueous and lipoid extracts of the latter.

Sabin and her associates[2] have reported the maturation of myeloblasts into myelocytes as a result of single transfusions in a patient with subacute myelogenous leukemia who had a large percentage of myeloblasts in the peripheral blood. In an effort to discover whether such a result could be due to substances hypothetically deficient in chronic myelogenous leukemia but present in the circulating blood of normal individuals, a third patient with typical chronic myelogenous leukemia, with, however, few myeloblasts in the peripheral blood, was given intravenously on each of 13 successive days about 320 cc. of citrated human plasma. The plasma was obtained by the centrifugalization, under sterile conditions, of blood obtained daily from normal individuals of the same blood group as the patient. No significant effect upon the blood values or differential myeloid count was produced during a period of 20 days. At the expiration of that time the patient was given daily for 5 days a transfusion of 500 cc. of citrated whole blood obtained from similar donors. The patient's red blood cells and hemoglobin were as a natural consequence increased, but during the period of the daily transfusions, and during a subsequent period of 17 days no significant changes in the white blood cell count or formula appeared.

Conclusions. 1. The oral administration of desiccated mucosa of the stomach and small intestine and of desiccated pancreas of the hog, or of mixtures of most of the fresh organs of newly born rabbits was without effect upon 2 patients with chronic myelogenous leukemia. 2. Daily transfusions of the plasma and of the whole blood of normal individuals were without effect upon the total or differential white blood cell counts of a third patient with chronic myelogenous leukemia. 3. No support for the idea that chronic myelogenous leukemia is a deficiency disease was obtained from these observations.

[1] Forkner, C. E., and Taylor, F. H. L., unpublished observations.

[2] Sabin, F. R., Austrian, C. R., Cunningham, R. S., and Doan, C. A., *J. Exp. Med.*, 1924, **40**, 845.

7808 C

Comparison of Wet and Dry Ashing Methods for Determining Blood Iron.

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Iron represents 0.335% by weight of the hemoglobin molecule.[1] Using this factor, blood hemoglobin may be estimated in grams per 100 cc. by the blood iron method as accurately as by the accepted oxygen capacity method.[2] Different standards representing 100% hemoglobin must be adopted for men,[3] women[3] and children.[4] The figure for the milligrams of iron per 100 cc. of blood may be converted into percentage hemoglobin by multiplying by 2 for men, by 2.25 for women, and by 2.5 for children.

Calibration of clinical hemoglobinometers such as the Dare and the Newcomer instruments is facilitated by using the blood iron method in preference to more complicated standardized methods. Simplification of procedures for determining blood iron even makes possible their use routinely in clinical laboratories. In view of these facts it is of utmost importance to have definite assurance of the accuracy of the method employed for the determination of blood iron.

We wish to report a comparative study of 2 different methods we have employed for blood iron in an effort to determine the normal range of this element in the blood of men, women, and children. One method involved the original Wong wet ashing procedure and the other method involved a dry ashing process.

Fowweather,[5] Smirk,[6] Kennedy,[7] Wong,[8] and others have reported slightly different wet ashing methods for determining blood iron in which the organic material is oxidized by an acid in combination with a strong oxidizing agent. The value of these methods lies in their simplicity and the speed with which the determinations may be made.

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[1] Butterfield, E. E., *Z. f. physiol. Chem.*, 1909, **62**, 173.

[2] Karshan, M., and Freeman, R. G., Jr., *J. Lab. and Clin. Med.*, 1929, **15**, 74.

[3] Sachs, A., Levine, V. E., and Appelsis, A., *Arch. Int. Med.*, 1933, **52**, 366; Sachs, A., Levine, V. E., and Fabian, A. A., *Ibid.*, in press.

[4] Sachs, A., Levine, V. E., and Fabian, A. A., unpublished communication.

[5] Fowweather, F. S., *Biochem. J.*, 1926, **20**, 93.

[6] Smirk, F. H., *Biochem. J.*, 1927, **21**, 36.

[7] Kennedy, R. P., *J. Biol. Chem.*, 1927, **74**, 385.

[8] Wong, S. Y., *J. Biol. Chem.*, 1928, **77**, 409.

In the original Wong procedure oxidation is carried on without the aid of external heat. We have modified the procedure by the application of heat to a flask containing blood to which have been added sulphuric acid and a saturated solution of potassium persulphate. Heating on the water-bath at 80°C. for 10 minutes causes a complete release of iron from the protein components of the red cell and insures more accurate results. Our figures for blood iron have been confirmed by other investigators who have used the Wong method.[9] Where other wet ashing methods have been used the figures did not agree with those which we obtained by the Wong procedure.[9, 10]

The other method involves dry ashing of the blood. Five cc. of oxalated blood were transferred to a vitreosil dish. The blood was dried on a hot plate with constant stirring to prevent spattering, and then ashed in an electric muffle for 8 hours at low red heat. To the ash was added 1 cc. of concentrated nitric acid, and the acid evaporated off on the hot plate. The procedure varies from this point if (a) blood copper and iron are to be estimated on the same sample and if (b) blood iron alone is to be determined.

(a) The ash is taken up with 3 cc. of 6N HCl and transferred to a centrifuge tube. The iron is precipitated as the hydroxide with 2 cc. of strong ammonia water. The tube is centrifugalized and the supernatant fluid containing the copper is poured off. For the determination of blood copper we employed the McFarlane[11] method. The iron determination is then continued as in (b).

(b) 4 cc. of concentrated sulphuric acid are added to 50 cc. of distilled water. Using this solution, the nitric acid treated ash or the ferric hydroxide obtained in (a) is dissolved and transferred to a 100 cc. flask. Two cc. of a saturated solution of potassium persulphate are added to the flask and the contents made up to the mark with distilled water. Five cc. of the solution in the flask are transferred to a 25 cc. graduated cylinder and 0.6 cc. of concentrated sulphuric acid added. The cylinder is cooled and made up to the 20 cc. mark with distilled water. A standard containing 0.1 mg. of iron and 0.08 cc. of concentrated sulphuric acid is made up to the 20 cc. mark in another cylinder. To the standard and unknown are added 1 cc. of the persulphate solution and 4 cc. of a 3N potassium thiocyanate solution, and comparison made colorimetrically.

Elvehjem[12] has reported a dry ashing method for determining

[9] Helmer, O. M., Emerson, C. P., Jr., *J. Biol. Chem.*, 1934, **104**, 157.

[10] Murphy, W. P., Lynch, R., and Howard, I. M., *Arch. Int. Med.*, 1931, **47**, 883.

[11] McFarlane, W. D., *Biochem. J.*, 1932, **26**, 1022.

[12] Elvehjem, C. A., *J. Biol. Chem.*, 1930, **86**, 466.

iron in biological materials in which the ash is boiled for an hour with strong alkali to convert to orthophosphates the pyrophosphates, which form at the temperature required for ashing. This step is necessary because pyrophosphates bind the iron in a complex molecule and thus prevent the union of ferric and thiocyanate ions. With some materials very rich in phosphates the color development is entirely inhibited unless measures are taken to eliminate or convert the phosphates. In blood, however, the problem of phosphate interference is of no great moment, since blood is relatively poor in phosphorus. If any pyrophosphate is present in the ash it tends to be converted to orthophosphate by the hot nitric acid which is added. Hot nitric acid converts metaphosphates and pyrophosphates to orthophosphates in experiments which we have performed.

We have made a series of 50 comparative determinations of blood iron by the modified Wong procedure and by our dry ashing method. We have found close agreement in the figures obtained when both methods were applied to the analysis of specimens of the same blood. The percentage deviation is about 1% for the whole series. This agreement justifies the use of our modification of the wet ashing procedure of Wong, which we have employed in the determination of the iron content of small samples of blood. It also justifies the use of the dry ashing method in a series of iron determinations we have just completed.

The recent reports[13] on copper in the blood with its special relation to anemia make it imperative at times to determine both the iron and copper content on the same blood. The wet ashing method is not suitable for the determination of both elements on the same sample of whole blood. The dry ashing method we have adopted has a great advantage, because it can be applied with convenience and accuracy to the simultaneous determination of iron and copper on the same blood sample. Using the dry ashing method for the determination of both iron and copper, we have made a study of bloods brought by one of us (L) from the Arctic in an effort to establish the incidence of anemia among Alaskan Eskimos.[14]

[13] Sachs, A., Levine, V. E., and Fabian, A. A., *Arch. Int. Med.*, in press.

[14] Sachs, A., Levine, V. E., and Fabian, A. A., unpublished communication.